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PRINCIPAL INVESTIGATOR: Dr. Peter H. Watson

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba, R3E-0W3 Canada

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13. ABSTRACT (Maximum 200 Words)

Invasion is a crucial component of the complex process of metastasis that marks the transition of breast cancer from local to life threatening disease. The approach we have taken to identify the molecular pathology underlying the onset of invasion, is to apply a combined microdissection and molecular approaches to a unique tissue bank resource. This enables us to isolate mRNA and directly compare gene expression profiles from pathologically defined regions of DCIS and early invasive tumor cells.

We have microdissected and identified a number of genes that show differential expression between DCIS and invasive components in 5 tumors by subtraction hybridization and 8 tumors by membrane filter cDNA array techniques. We have also pursued 2 specific genes, previously unexplored in relation to breast cancer, that show patterns of differential expression consistent with a role in the process of invasion - Psoriasin (S100A7) and Lumican, a small leucine-rich proteoglycan.

We conclude that our approach is productive in identifying novel genes that are previously unexplored in relation to early breast tumor progression and believe that these may provide markers of progression to invasive disease.

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THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Earl Grant Jr.

21 Feb. 2001

Table of Contents

"Identification of markers of the invasive phenotype in human breast cancer"

INTRODUCTION.

The management of Ductal Carcinoma in-situ (DCIS) depends on the estimation of the likelihood of recurrence as in-situ or invasive disease[1]. Recent morphological studies have provided useful potential improvements to older classifications of pre-invasive disease with more accurate prognostic significance, however there is clearly a need for better predicators of biological potential[2]. To address this critical issue we and others [3,4] have begun to search for molecular changes that may serve as such predictors. Our specific hypothesis is that alteration of gene expression is responsible for the progression of DCIS to invasive breast cancer and the acquisition of the invasive phenotype. Our approach has been to use an approach that can directly identify genes and alterations that occur in-vivo and that may contribute to the invasive phenotype, through microdissection of histologically defined components within single breast tumor sections and adaptation of established molecular subtraction techniques to accept small input RNA samples. The approach is made feasible by the unique design of our tumor bank resource [5-8].

BODY OF REPORT

The accomplishments over the third year of this award are detailed below in the context of the 2 major tasks defined in the statement of work.

TECHNICAL OBJECTIVE / AIM 1

1. 1 - 12, microdissect 12 tumors,

2. 3 - 15 complete 12 subtraction experiments (12 screens to detect genes showing loss of expression in DCIS relative to invasive components and 12 to detect genes with gain of expression),

3. 6 - 18   clone, sequence, and initiate screening of 100+ candidate genes by ISH and RT-PCR to confirm expression patterns,

Progress in year 3 (months 24-36):

Aim 1.1 & 1.2
- We have microdissected and completed the screening of 9 invasive tumors (6 new cases and 3 cases that were in progress at the time of last years progress report) for differential gene expression between DCIS and invasive components. These cases are in addition to 5 cases previously dissected and analysed using subtraction hybridization techniques in the first 2 years of the project, bringing the total number of microdissected and analysed cases through this project to 14 cases. All of the microdissected cases that have been analysed this year have been assessed using a membrane filter array method (Research Genetics Array filter GF200 combined with Pathways computer analysis software). This change in methodology reflects the recognition that gene array methods are more efficient than the previous techniques and allow simultaneous assessment of induced and repressed genes in each experiment.

Aim 1.3

- Amongst the recent 9 cases we have now identified a number of cDNA's that appear to be differentially expressed between adjacent in-situ and invasive elements within the same tumor. The change in methodology to membrane array methods allows direct positive identification of candidate genes without the necessity of cloning and sequencing unknown bands detected from subtraction methods. The mean number of differentially expressed genes is 81 (using a factor of 1.8 fold as the cutpoint for analysis). The individual cases show the following number of differential expressed cDNA's; Tumour #1: 37, Tumour #2: 36, Tumour #3: 56, Tumour #4: 66; Tumour #5: 103. Tumour #6: 151, Tumour #7: 119, Tumour #8: 74, Tumour #9: 119. Array images show comparative expression of genes in DCIS and Invasive components and a listing of specific cDNA's identified as differentially expressed in Tumor #3 (TB#11336) is appended as Figure 1A & 1B and Table 1 respectively. Further assessment to identify patterns of highly and/or consistently differentially expressed cDNA's in more than one in-situ/invasive tumor pair is now in progress as well as initiation of experiments to confirm differential expression of the current candidates by other techniques including in-situ hybridization and RT-PCR. However, our preliminary analysis of all 9 tumors indicates that there are 18 known genes and 15 ESTs that are differentially expressed in three or more of the tumours analyzed.

TECHNICAL OBJECTIVE / AIM 2

1. 18 - 24, determine expression patterns and relation to 'invasiveness' of a subset of genes isolated in tasks 1 to 3 in panels of pre-neoplastic, pre-invasive, invasive and metastatic breast lesions

2. 24 - 30, clone full-length cDNA's of a limited number of candidate invasion genes identified in task 4 and construct expression vectors

3. 30 - 36, create cell line models with overexpression of specific genes by stable transfection for future testing of the influence on invasion in in-vitro assay and nude mouse models

Over the past year (months 24-36);

Aim 2.1

- We have continued to study the biology of the psoriasin (S100A7) gene that we have previously identified as differentially expressed between in-situ and invasive carcinoma. This work has resulted in successful publication of work reported last year concerning the relation of psoriasin expression to prognostic parameters in invasive disease [9]. In pursuit of the possible intracellular role and interactions of psoriasin in breast cells, we reported last year the identification of 2 candidate interacting proteins through the use of a yeast 2-hybrid assay to screen a normal breast cell library. Over the past year we have continued to study one of these candidate proteins, RanBPM (selected on the basis that it showed the strongest interaction in the yeast assay).

- We have shown that RanBPM mRNA is expressed in both normal and neoplastic breast cell lines and have also analysed RanBPM mRNA expression by semi-quantitative RT-PCR in relation to psoriasin mRNA expression in a series of human breast tumors (n=80 cases) that represent a range of tumor types (35 invasive ductal, 25 invasive lobular, 17 special type with 5 medullary, 6 mucinous, 6 tubular). Our data shows that there are no significant differences in RanBPM and psoriasin expression with respect to tumor type, except the previously identified significant difference between DCIS and invasive tumors for psoriasin (Figure 3). However, both genes are correlated significantly with ER (but in opposing directions) and psoriasin and the psoriasin/RanBPM ratio also show significant associations with grade and inflammatory response within the tumor (Figure 4). These results indirectly support the hypothesis that RanBPM and Psoriasin may interact to have a joint biological effect, and indicate that this relates to ER status.

- Detailed analysis of RanBPM, Psoriasin and the Psoriasin/RanBPM ratio and correlation with specific tumor parameters shows that ER correlates with RanBPM (r=0.29, p=0.009), psoriasin (r=-0.3, p=0.006) and Psoriasin/RanBPM ratio (r= -0.39, p=0.0003) , (Spearman test), Grade correlates with

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psoriasin (r=0.33, p=0.016) and Psoriasin/RanBPM (r=.299, p=.03), but not with RanBPM (Spearman test), Inflammation correlates with psoriasin (r=0.385, p=0.001) and Psoriasin/RanBPM ratio (r=.42, p=0.0003) (Spearman test). Also analysis of RanBPM, Psoriasin and the Psoriasin/RanBPM ratio and relation with specific tumor parameters assessed as categorical variables (Mann Whitney tests) shows that a) Tumor Types: there is no significant difference in RanBPM or psoriasin between 5 tumor types, b) Grade; grade l/m/h; ANOVA, ns (psor 0.083), c) ER status; ER- vs ER+ve; Ran p=0.0156, psoriasin p=0.0283, Psoriasin/RanBPM ratio p=0.004, d) Inflammation: infl low vs high; ran ns, psoriasin p=0.01, Psoriasin/RanBPM p=0.0027.

- We have also worked to establish that the interaction seen in yeast between psoriasin and RanBPM is also present in human and breast cells in-vivo. Our attempts to demonstrate an interaction by co-immunoprecipitation has been thwarted by the fact that we do not yet have an anti-RanBPM antibody to directly detect the protein. We therefore initially cloned RanBPM into a Flag-tag expression vector (Sigma). However even though in-vitro transcription and translation performed using this vector is successful and generates an appropriate sized protein, we have not been able to detect the C terminal Flag-tagged protein in transiently transfected cells. We have concluded that the problem is probably related to internalization of the c-terminal Flag tag within the folded protein in-vivo, and are now in the process of recloning the RanBPM cDNA into a different N-terminal tag vector in order to pursue co-immunoprecipitation experiments with psoriasin.

- In the meantime, we have also used confocal immunofluorescent microscopy to localize the psoriasin protein within human breast cells. Our results show that psoriasin protein is predominantly localized to a region adjacent to the nucleus (but is also present in the nucleus and cytoplasm) and that psoriasin co-localizes with pericentrin, which is a known centrosomal protein (Figure 5). While we have not yet been able to examine RanBPM in the same cells, these results are highly consistent with the notion that psoriasin and RanBPM are present at the centrosome and that psoriasin interacts with RanBPM.

- We have also continued to work on the lumican and related decorin genes. We have successfully published work that was reported last year concerning the relative patterns of expression of lumican

and decoring within breast tissues [10]. We have also selected a series of 178 node negative breast cancer cases that were treated uniformly by tamoxifen +/- radiotherapy to examine the relation of lumican expression to stromal composition and outcome. We have worked to develop an infra-red spectroscopy assay (in collaboration with Dr M Jackson, NRC) to measure stromal collagen content and composition as well as Western blot and immunohistochemistry assays to assess EGFR status.

Aim 2.3

- We reported previously our work generating 2 MDA-231^{psoriasin} transfected cell line models and the finding that psoriasin overexpression has no significant effect on the growth or invasiveness of this already invasive breast tumor cell line. Therefore we now intend to assess the effect of overexpression of psoriasin in a pre-invasive cell line, the MCF10AT model and reported last year the successful generation of a single MCF10AT^{psoriasin} transfected cell clone. In the past year we have made limited progress on this aspect of our studies, however repeated transfections of the CMV-psoriasin into MCF-10AT3B cells (using zeocin as a marker since they have previously been transfected with ras-neo) have provided us with a second MCF10AT^{psoriasin} clone with overexpression detected by northern and western blot. In the next year we plan to use these 2 cell line models to conduct in-vitro assessment of the effect of psoriasin on growth and invasiveness in a non-invasive cell line.

KEY RESEARCH ACCOMPLISHMENTS

1. Microdissection and screening of a further 9 tumors by membrane array technique, and identification of 731 differentially expressed cDNA's in aggregate, and amongst these cases 33 cDNAs that are consistently differentially expressed and are therefore potential candidates for further study as invasion related genes.

2. Establishing that psoriasin protein can be detected and localized by confocal microscopy to the centrosome in human cells, consistent with our data suggesting that psoriasin interacts with centrosomal proteins and our hypothesis that psoriasin influences invasion

3. Exploring the expression of a potential interacting protein and showing that this protein is indeed expressed in human breast tumor cells and tissues and that an increase in the ratio of psoriasin to this protein occurs and is significantly correlated with low ER, high tumor grade and increased inflammation.

REPORTABLE OUTCOMES

1. Papers

   - S. Al Haddad, Z. Zhang, E. Leygue, L. Snell, A. Huang, Y. Niu, T. Hiller-Hitchcock, K. Hole, L. C. Murphy, and P. H. Watson. Psoriasin (S100A7) expression and invasive breast cancer. *Am.J.Pathol.* 155 (6):2057-2066, 1999.

   - Leygue E, Snell L, Dotzlaw H, Hole K, Hiller-Hitchcock T, Murphy LC, Roughley PJ, Watson P,. "Lumican and decorin are differentially expressed in human breast carcinoma" in press Oct 2000, J Pathology

2. Abstract

   - Ethan D. Emberley, A. Kate Hole, R. Daniel Gietz, Leigh C. Murphy and Peter H. Watson. Interaction of the Differentially Expressed S100A7 Gene With Centrosomal Proteins. Submitted to San Antonio Breast cancer conference, Dec 2000.

3. Funding

   Operating Grants awarded this year based in part on data generated through this USArmy IDEA grant;

   - National Cancer Institute of Canada/Canadian Breast Cancer Research Initiative (NCIC/CBCRI), "The role of Psoriasin in progression of early breast cancer", $109,000 pa, 2000-2003

   - Canadian Institutes of Health Research (CIHR/MRC), "The role of the small leucine rich proteoglycan in human breast cancer", $120,000pa, 2000-2003

   - NCIC/CBCRI Streams of Excellence, group grant, "The role of extracellular matrix in mediating risk of breast cancer", $60,000 pa, 2000-2003

CONCLUSIONS

Importance & Implications: This will lead to the identification of biological markers and cellular alterations that are directly relevant to early progression to invasive disease. This knowledge will in turn ultimately contribute to clinical markers to predict progression of early breast cancer and strategies to improve on current treatments for early invasive disease.

Assessment and Future strategy:

Although we are pleased with our overall progress, and in particular with the completion of aim 1.1 and 1.2 with a total of 13 cases microdissected and the continued progress with aim 2 focusing on the study of 2 candidate 'invasion' related genes (psoriasin and lumican), our momentum has been slower than it might have been. This is largely related to the fact that a key member on the project, Ms Kate Hole (Research Associate) who was responsible for the subtraction and array analyses, was away for a 6 month period of maternity leave in 1998/99 and then shortly after returning to the project decided to leave my laboratory as of March 2000. This has left a key position open and it was initially difficult to offer an attractive position to a new research associate with just 7 months remaining til the end to the project. However, in view of this we have applied for and have now recently been granted a one year extension to the project. We hope to now be able to complete the analysis of our array data from the most recent set of 8 cases and to continue to screen the most promising candidate genes that emerge as reproducibly differentially expressed in more than one case.

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REFERENCES

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10. Leygue E, Snell L, Dotzlaw H, Hole K, Hiller-Hitchcock T, Murphy LC, Roughley PJ, Watson P. "Lumican and decorin are differentially expressed in human breast carcinoma" in press, J Pathology 2000

APPENDICES

Figures

- Table 1 (2 pages)

- Figures 1-4 (5 pages)

Papers/abstracts

- S. Al Haddad, et al. Psoriasin (S100A7) expression and invasive breast cancer. *Am.J.Pathol.* 155 (6):2057-2066, 1999. (10 pages)

- Leygue E et al "Lumican and decorin are differentially expressed in human breast carcinoma" in press, J Pathology 2000. (9 pages)

- Ethan D. Emberley, A. Kate Hole, R. Daniel Gietz, Leigh C. Murphy and Peter H. Watson. Interaction of the Differentially Expressed S100A7 Gene With Centrosomal Proteins. Submitted to San Antonio Breast cancer conference, Dec 2000. (1 page)

## Table 1. Overexpressed in DCIS vs Matched Invasive Component

field	grid	row	col	cluster id	cdna id	acc	title	chro	ratio	diff
1	A	12	8	Hs.75708	323506	W45690	Ribosomal protein, large, P1	15	2.37	515.83
1	A	13	9	Hs.4059	80910	T70098	Human neutral amino acid transporter B mRNA, complete cds	-	3.12	654.09
1	A	14	8	Hs.75459	66686	T67270	Ribosomal protein S3	X	2.94	2737.87
1	A	27	11	Hs.74623	950680	AA6085	Damage-specific DNA binding protein 1 (127 kD)	11	1.97	559.44
1	B	3	12	Hs.46437	842860	AA4863	Cytokine receptor family II, member 4	21	2.04	1009.61
1	B	17	9	Hs.40403	781510	AA4321	Human melanocyte-specific gene 1 (msg1) mRNA, complete cds	X	2.02	879.86
1	C	14	2	Hs.29797	66686	T67270	UBIQUINOL-CYTOCHROME C REDUCTASE COMPLEX SUBUNIT VI REQUIRING PROT	X	1.89	783.82
1	C	20	10	Hs.46925	685912	AA2625	Human EYA3 homolog (EYA3) mRNA, complete cds	-	1.92	1571.34
1	D	9	12	Hs.111572	66560	T67053	Human rearranged immunoglobulin lambda light chain mRNA	22	4.77	2300.88
1	D	10	9	Hs.12409	39593	R51912	Human somatostatin I gene and flanks	3	2.42	866.57
1	D	11	4	Hs.11711	233078	H75699	Human mRNA for KIAA0297 gene, partial cds	14	3.54	543.61
1	D	13	6	Hs.78683	525518	AA0646	H.sapiens mRNA for herpesvirus associated ubiquitin-specific protease (HAUSP)	-	2.35	1916.47
1	E	1	12	Hs.86358	897770	AA5985	Cellular retinoic acid-binding protein 2	1	1.91	7717.27
1	E	2	5	Hs.26232	183462	H45455	Human alpha-mannosidase (6A8) mRNA, complete cds	-	3.26	579.59
1	E	2	7	Hs.38481	214572	H73724	Cyclin-dependent kinase 6	7	2.18	759.47
1	E	9	2	Hs.84232	823864	AA4906	Transcobalamin II	22	1.82	574.43
1	E	13	6	Hs.90370	823930	AA4902	H.sapiens mRNA for Sop2p-like protein	17	2.41	3906.12
1	E	28	10	Hs.47075	767345	AA4185	Human (clone 8B1) Br-cadherin mRNA, complete cds	5	1.91	3304.52
1	F	2	6	Hs.12272	771084	AA4273	Homo sapiens GT197 partial ORF mRNA, 3' end of cds	17	2.38	6358.36
1	F	7	5	Hs.326	756488	AA4364	Human TAR RNA binding protein (TRBP) mRNA, complete cds	-	1.86	1017.52
1	F	18	5	Hs.76887	127519	R08876	Human 26S proteasome-associated pad1 homolog (POH1) mRNA, complete cds	-	1.98	1647.91
1	G	12	12	Hs.108181	207358	H58873	Human (HepG2) glucose transporter gene mRNA, complete cds	1	2.27	691.85
1	H	2	12	Hs.25856	739183	AA4212	CD68 antigen	-	2.34	1587.27
1	H	4	7	Hs.23118	275738	R93176	Carbonic anhydrase I	8	3.09	3704.46
1	H	4	8	Hs.79127	491066	AA1369	SERINE/THREONINE-PROTEIN KINASE RECEPTOR R1 PRECURSOR	2	2.34	1438.40
1	H	11	6	Hs.25180	177737	H46663	H.sapiens Pur (pur-alpha) mRNA, complete cds	-	2.60	798.39
1	H	11	7	Hs.75183	179403	H50500	CYTOCHROME P450 IIE1	10	2.10	2508.93

| field	grid	row	col	cluster id	cdna id	acc	title	chro	ratio	diff
1	H	11	8	Hs.43910	898198	AA5985	PUTATIVE MUCIN CORE PROTEIN PRECURSOR 24	6	3.42	1614.55
1	H	12	10	Hs.82765	897806	AA5985	Human MOP1 mRNA, complete cds	14	2.10	1419.41
1	H	12	12	Hs.77479	275180	R84893	Homo sapiens Chromosome 16 BAC clone CIT987-SKA-589H1 ~complete genomic seque	16	2.93	822.61
1	H	14	11	Hs.82032	843140	AA4859	ER LUMEN PROTEIN RETAINING RECEPTOR 2	7	1.83	2946.50
2	A	4	11	Hs.50454	296838	W01211	ESTs	-	4.37	609.83
2	B	25	5	Hs.16724	161195	H25229	ESTs	-	4.35	1020.13
2	C	26	3	Hs.113052	125148	R05309	ESTs, Highly similar to HYPOTHETICAL 39.5 KD PROTEIN C12G12.06C IN CHROMOSO	-	2.66	1068.62
2	C	30	11	Hs.93231	322723	W15465	ESTs	8	1.87	1931.63
2	D	6	10	Hs.38901	212787	H70114	ESTs	-	2.57	750.36
2	D	18	3	Hs.117884	204737	H57309	ESTs, Highly similar to SNAIL PROTEIN HOMOLOG [Xenopus laevis]	-	2.73	535.17
2	D	21	3	Hs.35752	232946	H75599	ESTs, Highly similar to SERINE/THREONINE-PROTEIN KINASE SGK [Rattus norvegicus]	-	4.33	839.11
2	D	22	3	Hs.10325	141959	R67562	ESTs, Highly similar to RSP5 PROTEIN [Saccharomyces cerevisiae]	8	1.90	3925.21
2	D	26	11	Hs.93345	214614	H73661	ESTs	-	1.91	569.98
2	D	27	6	Hs.20181	127766	R08690	ESTs	-	1.88	618.02
2	D	27	8	Hs.34192	194965	R88734	ESTs	-	2.99	707.74
2	E	18	11	Hs.82223	485854	AA0404	ESTs	-	2.88	605.83
2	E	23	12	RG.47	358531	W96155	-	-	1.93	1875.01
2	E	24	3	Hs.119142	825470	AA5043	ESTs, Highly similar to PUTATIVE GTP-BINDING PROTEIN MOV10 [Mus musculus]	17	2.60	695.36
2	F	17	10	Hs.44970	243878	N45263	ESTs	-	1.93	3456.04
2	F	22	3	Hs.95321	204083	H55893	ESTs, Highly similar to PRE-MRNA SPLICING FACTOR RNA HELICASE PRP28 [Sacchar	-	2.01	815.61
2	F	24	10	Hs.49688	292908	N90968	ESTs	-	4.30	1135.42
2	G	27	3	Hs.94306	196005	R91904	ESTs, Highly similar to AQUAPORIN 3 [Rattus norvegicus]	9	3.53	787.75
2	G	29	4	Hs.119606	38465	R49470	ESTs	-	1.92	3035.68
2	G	29	9	Hs.39832	240406	H78083	ESTs	-	2.04	1428.22
2	H	6	7	Hs.23630	131563	R24223	ESTs	-	2.22	4329.16
2	H	16	5	Hs.16037	123433	R00591	ESTs	4	2.24	615.45
2	H	18	12	Hs.107286	284592	N76193	ESTs	11	2.24	2619.20
2	H	20	9	Hs.37926	210548	H65052	ESTs	11	1.84	1539.80
2	H	23	5	Hs.16222	242070	H94236	ESTs	-	2.55	513.08

FIGURE 1A

Filter Array's of Matched DCIS and INVASIVE components within a single breast tumor

DCIS   INVASIVE

1   2   3   4   1   2   3   4
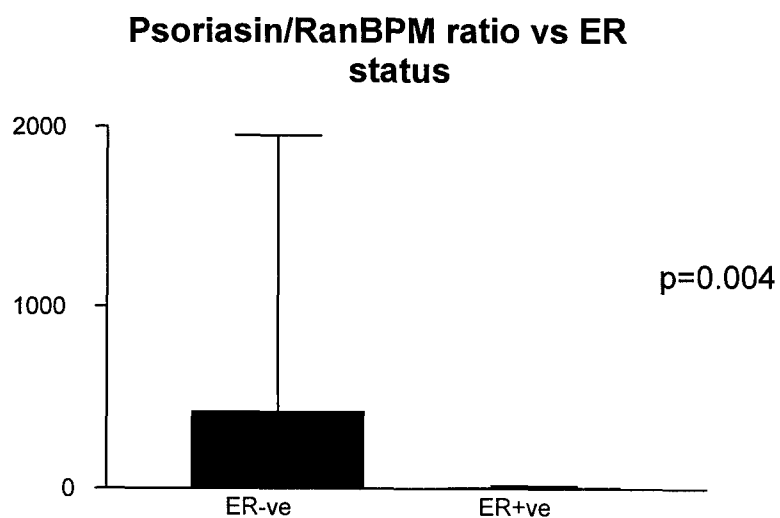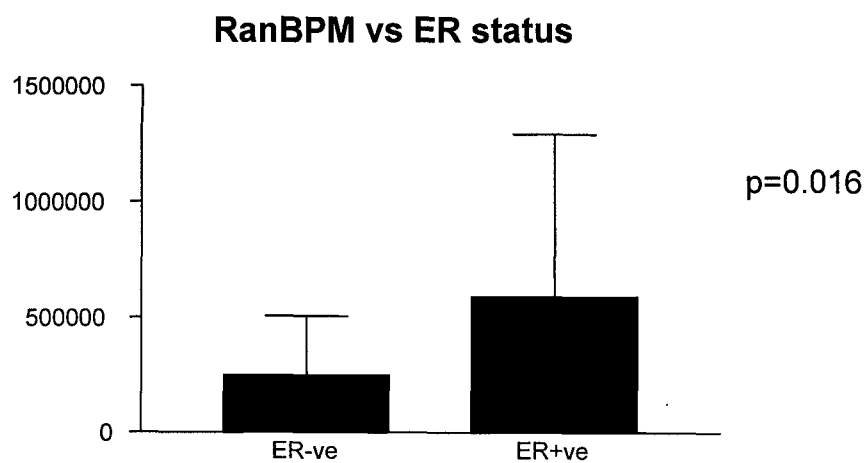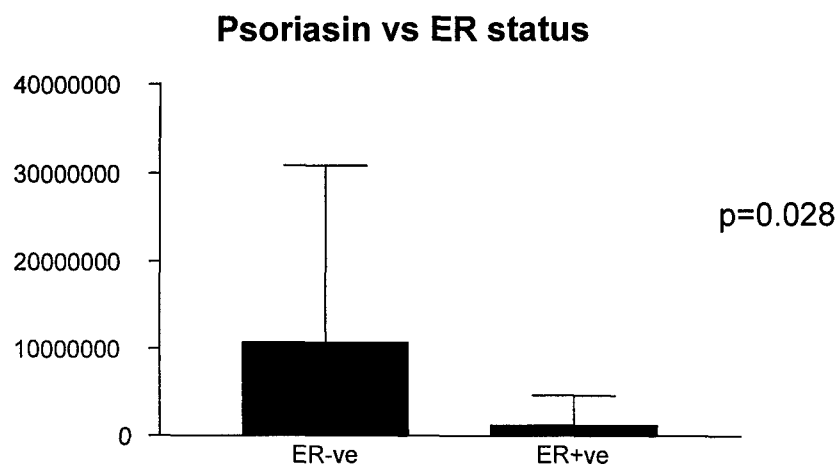

5   6   5   6
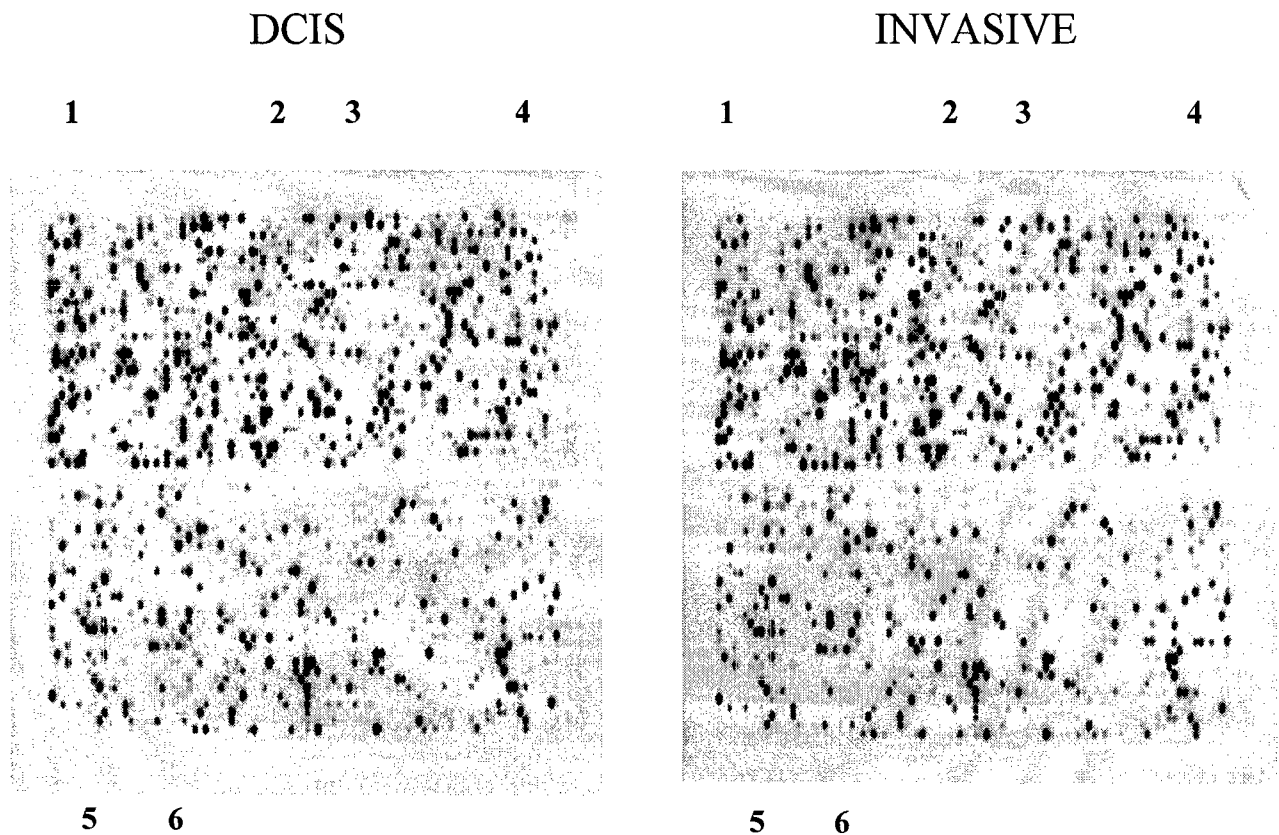
FIGURE 1A. RNA from DCIS and INVASIVE tumour tissue, microdisected from a single patient, was labelled with ^{33}P and hyrbidized to G200 filters from Research Genetics (matched lot #s). Above are the phosphoimages obtained.
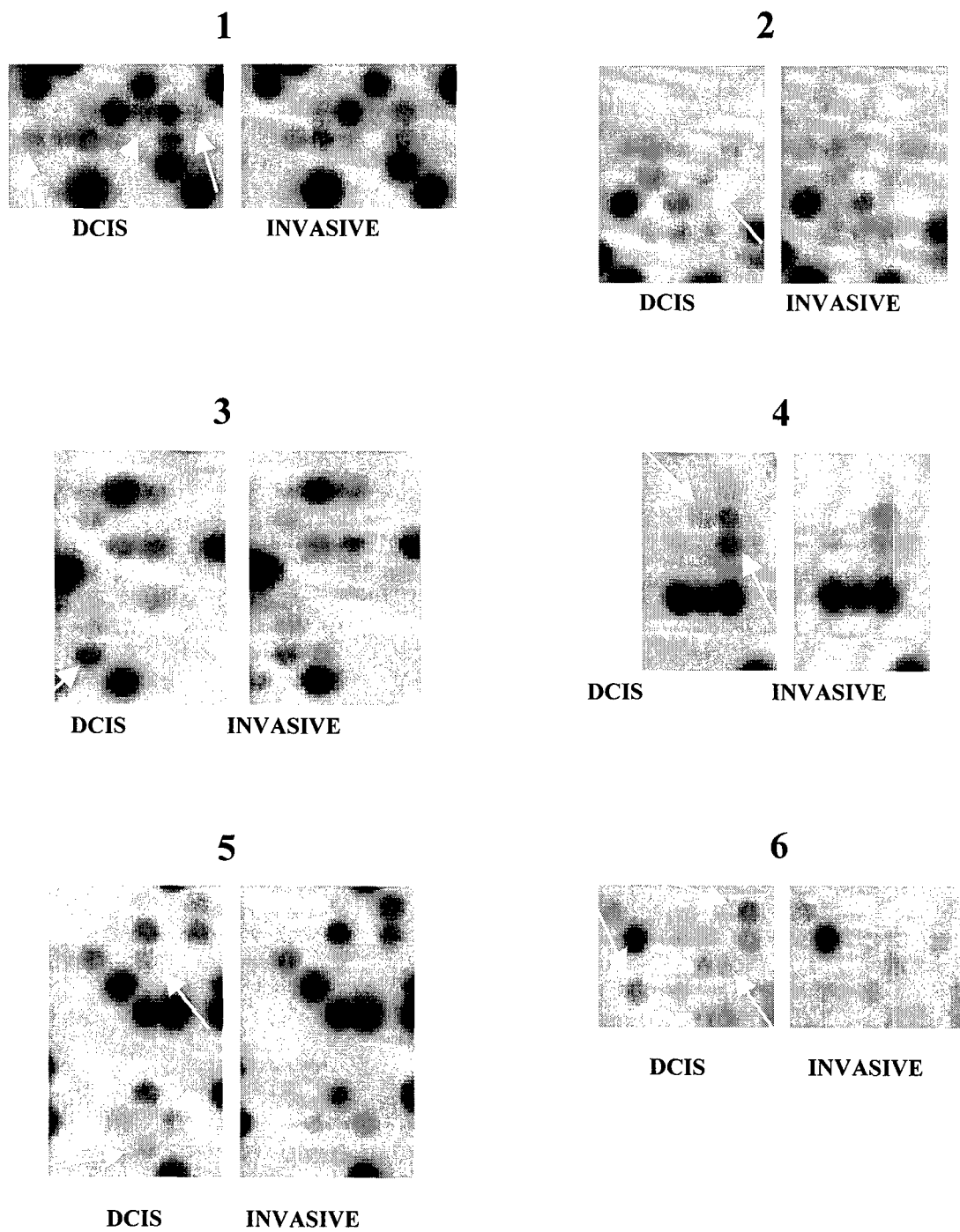
Figure 1B. Magnified regions of the images shown in figure 1A .showing differential
expression of specific cDNA's within the DCIS and INVASIVE components
(identified by the arrows in the DCIS panel of each region)
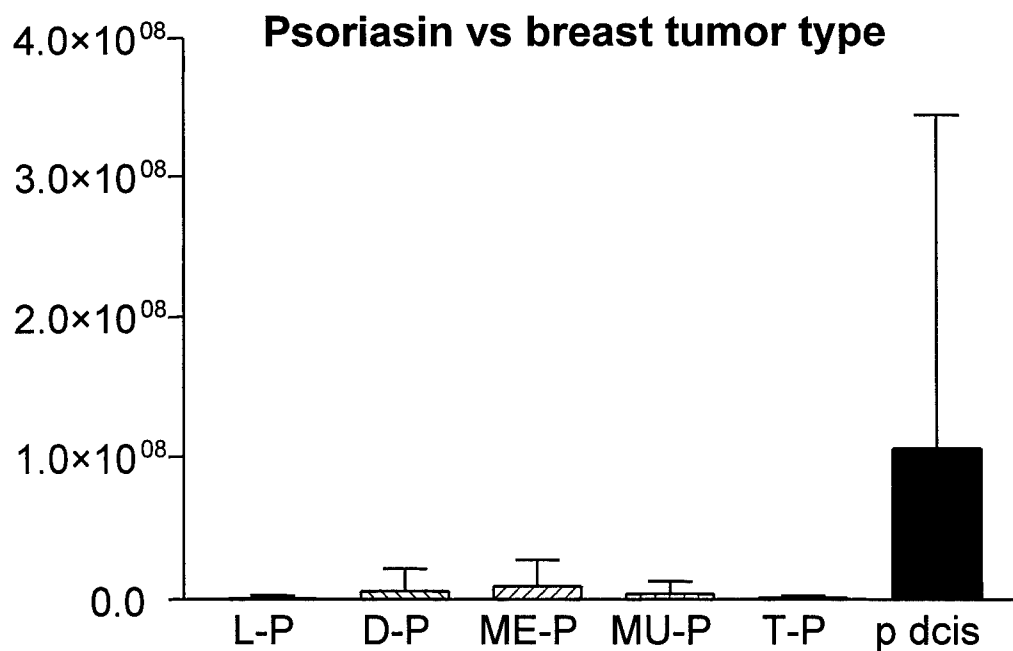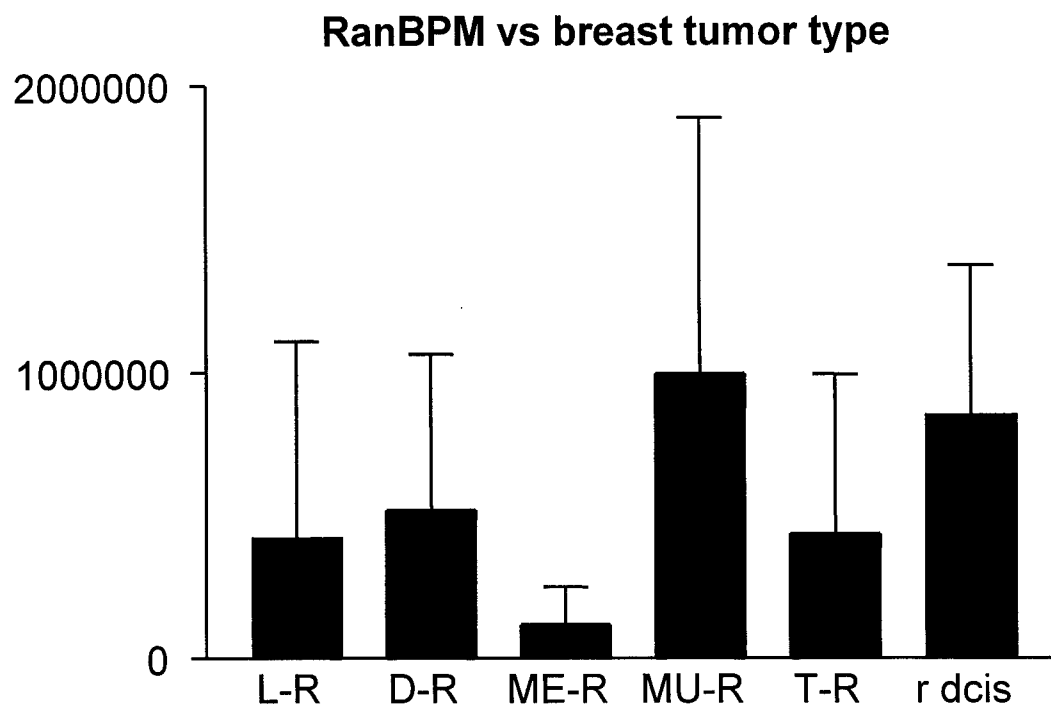
Figure 2. Relationship between expression of RanBPM and Psoriasin and Tumor Type (L=lobular, D=ductal, ME=medullary, MU=mucinous, T=tubular, dcis=ductal carcinoma in-situ). Bar=Mean + SD

Psoriasin vs ER status

p=0.028

RanBPM vs ER status

p=0.016

Psoriasin/RanBPM ratio vs ER status

p=0.004

Figure 3. Relationship between expression of RanBPM, Psoriasin and the Psoriasin/RanBPM ratio with ER status (-ve or +ve). Bar=Mean + SD

Co-localization of psoriasin and pericentrin in human breast cells
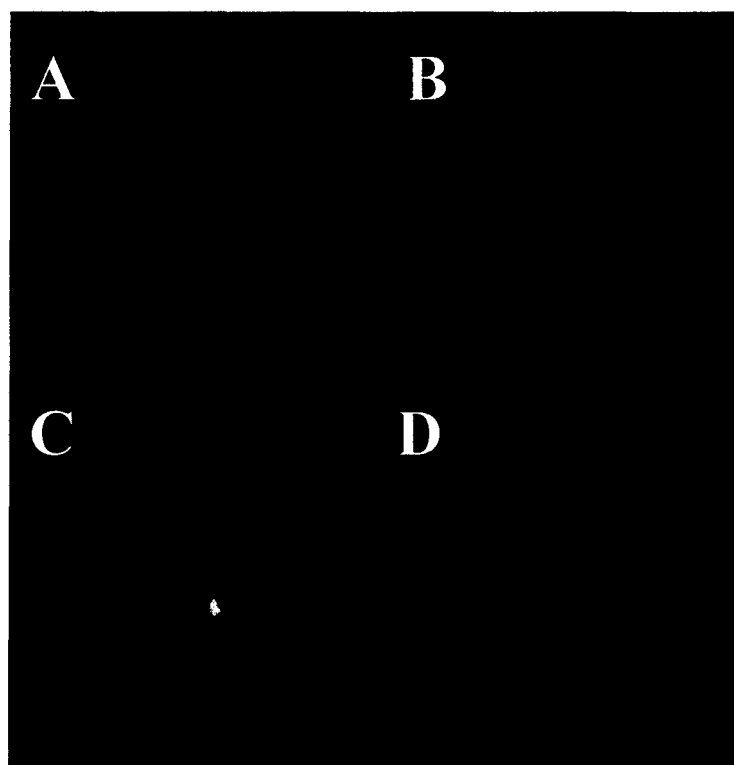
Figure 4. Confocal Immunoflorescence showing cellular localization of (panel A)
psoriasin (FITC), and (panel B) pericentrin (Cy5) in MDA-MB-231cells stably
transfected with psoriasin expression vector (clone FA1). Panel (C) shows overlay
of images (A) and (B). Panel (D) is the equivalent of panel (C) with a control MDA-
MB-231 cell line (VA1) that is stably transfected with the empty expression vector
showing only pericentrin signal.

Psoriasin (S100A7) Expression and Invasive Breast Cancer

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Alteration of psoriasin (S100A7) expression has previously been identified in association with the transition from preinvasive to invasive breast cancer. In this study we have examined persistence of psoriasin mRNA and protein expression in relation to prognostic factors in a cohort of 57 invasive breast tumors, comprising 34 invasive ductal carcinomas and 23 other invasive tumor types (lobular, mucinous, medullary, tubular). We first developed an IgY polyclonal chicken antibody and confirmed specificity for psoriasin by Western blot in transfected cells and tumors. The protein was localized by immunohistochemistry predominantly to epithelial cells, with both nuclear and cytoplasmic staining, as well as occasional stromal cells in psoriatic skin and breast tumors; however, *in situ* hybridization showed that psoriasin mRNA expression was restricted to epithelial cells. In breast tumors, higher levels of psoriasin measured by reverse transcriptase-polymerase chain reaction and Western blot (93% concordance) were significantly associated with estrogen and progesterone receptor-negative status ($P < 0.0001$, $P = 0.0003$), and with nodal metastasis in invasive ductal tumors ($P = 0.035$), but not with tumor type or grade. Psoriasin expression also correlated with inflammatory infiltrates (all tumors excluding medullary, $P = 0.0022$). These results suggest that psoriasin may be a marker of aggressive behavior in invasive tumors and are consistent with a function as a chemotactic factor. *(Am J Pathol 1999, 155:2057–2066)*

Earlier diagnosis of breast cancer has increased the need for the identification of molecular alterations that might serve as tissue markers to predict the risk of progression to metastatic disease. Among the most important of these alterations are likely to be those associated with the development of the invasive phenotype and the transition from preinvasive to invasive cancer with the capability for subsequent metastasis.

We have recently identified psoriasin (S100A7) as a gene that is frequently overexpressed in preinvasive ductal carcinoma *in situ* (DCIS) relative to adjacent invasive carcinoma, suggesting a role in breast tumor progression.[1] Other members of the S100 gene family of calcium-binding proteins have been implicated in a range of biological processes, including tumor metastasis.[2] In particular, S100A2 has been shown to be down-regulated in breast tumor cells relative to their normal epithelial cell counterparts,[3] whereas up-regulation of S100A4 has been strongly implicated in breast tumor metastasis.[4-6] In the latter case this may reflect the ability of S100A4 to influence cell motility,[7] the cytoskeleton[6,8,9] or cell adhesion molecules.[10] Psoriasin was initially identified as a highly abundant protein belonging to the S100 gene family,[11] expressed by abnormally proliferating keratinocytes in psoriatic epidermis.[12,13] It has subsequently been shown to be a secreted protein that can exert an effect as a chemotactic factor for inflammatory cells.[14,15] However, the function of psoriasin in breast cancer remains to be determined.[16] In this study we have developed a psoriasin-specific antibody and evaluated the persistence of psoriasin expression in invasive breast tumors with different invasive and metastatic potential as well as host inflammatory response.

Materials and Methods

Human Breast Tissues and Cell Lines

All breast tumor cases used for this study were selected from the NCIC–Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As has previously been described,[17] tissues accrue to the Bank from cases at multiple centers within Manitoba and are rapidly collected and processed to create matched formalin-fixed embedded and frozen tissue blocks for each case, with mirror-image surfaces

oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted in hematoxylin/eosin (H&E)-stained sections from the face of the paraffin tissue block by a pathologist. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for the selection of specific paraffin and frozen blocks from cases for this study. For each case interpretations included an estimate of the cellular composition (including the percentage of invasive epithelial tumor cells, collagenous stroma, and fatty stroma), tumor type, and tumor grade for ductal tumors (Nottingham score).[18,19] The inflammatory host response was scored semiquantitatively on a scale of 1 (low) to 5 (high). Steroid receptor status was determined for all cases by ligand binding assay performed on an adjacent portion of tumor tissue. Tumors with estrogen and progesterone receptor levels above 20 fmol/mg and 15 fmol/mg of total protein, respectively, were considered ER- or PR-positive.

Two cohorts of tumors were selected. The first cohort comprised 35 invasive ductal carcinomas selected to include six subgroups differing with respect to estrogen receptor status (ER-positive and ER-negative) and tumor grade (low, intermediate, high). Additional selection criteria also included high tissue quality, presence of invasive tumor within >35% of the cross section of the frozen block for invasive ductal cases, and minimal (<5%) normal or *in situ* epithelial components. The second cohort comprised 23 invasive tumors selected to include four subgroups of different tumor types[18] that vary in differentiation and metastatic potential, including invasive lobular (six), medullary (five), tubular (six), and colloid (six). Similar secondary criteria were also used for this cohort.

For analysis of antibody specificity and for positive controls for tumor assays, MCF7 human breast cancer cells obtained from the American Type Culture Collection (Manassas, VA) were used. MCF7 cells were grown as previously described under normal conditions in the presence of 5% fetal bovine serum, to provide a negative control.[20] Alternatively MCF7 cells were subjected to estrogen-deprived conditions in the presence of charcoal-stripped serum before stimulation by estradiol (10^{-8} mol/L) for 48 hours before harvesting to induce psoriasin expression and provide a positive control. As an additional positive control MDA-MB-231 human breast cancer cells were transfected with a plasmid containing the cytomegalovirus (CMV) promotor adjacent to the psoriasin cDNA (Hiller-Hitchcock T, Leygue E, Cummins-Leygue C, Murphy LC, Watson PH, manuscript in preparation), and stable transfectants (CL7FD3 cell clone) expressing psoriasin mRNA were also used.

Antibody Reagents

A psoriasin-specific chicken IgY polyclonal antibody was generated by immunization of chickens with a 14-amino acid peptide corresponding to the carboxy terminus of psoriasin (KQSHGAAPCSGGSQ; Bionostics, Toronto, and Aves Labs). A >90% pure IgY fraction from chicken egg yolk was obtained in phosphate-buffered saline

(PBS) and then further purified by passing it over a psoriasin peptide affinity column made by binding the synthetic peptide to N-hydroxy-succinimide-activated Sepharose 4B (Pharmacia Biotech), according to the manufacturer's instructions. The bound IgY was then eluted with 5.0 mol/L sodium thiocyanate, followed by dialysis against PBS. Additional antibodies used included a commercial anti-S100 antibody (Sigma, St. Louis, MO) as well as a rabbit polyclonal antibody, raised against the recombinant protein (kindly provided by Prof J. Celis, University of Aarhus, Aarhus, Denmark).

Western Blot Analysis

For tumors, multiple sections ($10-20 \times 20 \ \mu m$) were cut from the face of frozen tissue blocks immediately adjacent to the face of the matching paraffin block.[17] For cell lines, trypsinized cell pellets were obtained from breast cancer cell lines (grown to ~80% confluence). Total protein lysates were extracted from both the cell line pellets and frozen tissue sections, using Tri-reagent (Sigma), as described by the manufacturer. The recovered protein was dissolved in SDS isolation buffer (50 mmol/L Tris, pH 6.8, 20 mmol/L EDTA, 5% sodium dodecyl sulfate (SDS), 5 mmol/L β-glycerophosphate) and a cocktail of protease inhibitors (Boehringer Mannheim, Laval, PQ). Protein concentrations were determined using the Micro-BCA protein assay kit (Pierce, Rockford, IL). Sixty micrograms of total protein lysates were run on a 16.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mini gel, using Tricine SDS-PAGE to separate the proteins,[21] and then transferred to 0.2-μm Nitrocellulose (Bio-Rad, Mississauga, ON). After blocking in 10% skimmed milk powder in Tris-buffered saline-0.05% Tween (TBST buffer), blots were incubated with chicken IgY anti-psoriasin antibody (~15 μg/ml in TBST), followed by incubation with secondary antibody, rabbit IgG anti-chicken IgY conjugated to horseradish peroxidase (1:5000 dilution in TBST; Jackson ImmunoResearch Laboratories), and visualization by incubation with Supersignal (Pierce), per the manufacturer's instructions. Exposed x-ray films were photographed, and the band intensities were determined by video image analysis, using MCID M4 software (Imaging Research, ST. Catherine's, ON). All signals were adjusted with reference to the psoriasin-transfected MDA-MB-231 cell control (CL7FD3), run on each blot.

Immunohistochemistry

Immunohistochemistry was performed on 5-μm paraffin-embedded breast tumor tissue sections from tissue blocks fixed in 10% neutral buffered formalin for 18-24 hours. After deparaffinizing, clearing, and hydrating to PBS buffer (pH 7.4) containing 0.05% Tween 20 (Mallinckrodt), the sections were pretreated with hydrogen peroxide (3%) for 10 minutes to remove endogenous peroxidases, and nonspecific binding was blocked with normal rabbit serum (1:50; Sigma). Primary chicken IgY

anti-psoriasin antibody (1:500 dilution in PBS) was applied for 1 hour at 37°C followed by washing and incubation with the secondary antibody, peroxidase-conjugated affinity purified rabbit anti-chicken (1:200 dilution), for 1 hour at room temperature. Detection was performed with 3,3′-diaminobenzidine tetrahydrochloride peroxidase substrate (Sigma) and counterstaining with methyl green (2%), followed by dehydration, clearing, and mounting. A positive tissue control and a negative reagent control (normal rabbit serum only/no primary antibody) were run in parallel in all experiments. Immunostaining was scored semiquantitatively by assessing the average signal intensity (on a scale of 0 to 3) and the proportion of tumor cells showing a positive nuclear signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0 greater than one-half). The intensity and proportion scores were then multiplied to give an overall score, and tumors with a score equal to or higher than 1.0 were deemed positive.

In Situ Hybridization

In situ hybridization was performed on paraffin sections (5 µm) according to a previously described protocol.[1] Linearized psoriasin plasmid cDNA (1.0 µg/µl) was used to generate UTP^{S35}-labeled sense and antisense RNA probes with the Riboprobe System (Promega, Madison, WI) according to the manufacturer's instructions. Sense and antisense probes were equalized by diluting 1×10^6 cpm/µl in hybridization solution. These were then applied to paraffin sections (approximately 30 µl of probe per section) that had undergone postfixation with 4% paraformaldehyde (pH 7.4) in PBS and further pretreatments with triethanolamine/acetic anhydride and proteinase K before hybridization. Sections were then coverslipped, sealed, and incubated overnight in a humid chamber at 42°C. After coverslip removal, sections underwent incubation in posthybridization solution and buffered RNase A (20 µg/ul), followed by several washes in descending dilutions of standard saline citrate buffer to remove weakly bound nonspecific label. After dehydration in ethanol containing 300 mmol/L ammonium acetate, the sections were coated in NTB-2 Kodak emulsion, subsequently developed after various time intervals from 2 to 5 weeks, and counterstained with Lee's methylene blue and basic fuchsin. Psoriasin expression was assessed by bright-field microscopic examination at low power (10× objective) magnification with reference to the negative sense and positive control tumor sections run with each batch. Levels were scored semiquantitatively as previously described[22] by assessing the average signal intensity (on a scale of 0 to 3) and the proportion of tumor cells showing a positive signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0 greater than one-half). The intensity and proportion scores were then multiplied to give an overall score, and tumors with a score equal to or higher than 1.0 were deemed positive.

Reverse Transcriptase-Polymerase Chain Reaction Analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed based on extracted RNA (600 ng) that was reverse transcribed in a total volume of 20 µl as described previously.[1] Briefly, reverse transcription was completed with the following reaction mixture: for each sample, 200 ng (2 µl of 0.1 µg/µl) of total RNA was added to 16 µl of RT mix (4 µl of 5× RT buffer; 1 µl of each of dATP, dCTP, dGTP, and dTTP, all at 2.5 mmol/L; 2 µl of 0.1% bovine serum albumin; 2 µl of 0.1 mol/L dithiothreitol; 1 µl of 0.25 mol/L random hexamer primer; 2 µl of dimethyl sulfoxide (DMSO), and 1 µl of 200 units/µl of Moloney murine leukemia virus reverse transcriptase) and incubated at 37°C for 1.5 hours. Each PCR was performed in 50-µl volume, using 1 µl of the completed RT reaction (cDNA); 30.8 µl of sterile water; 5 µl of 10× PCR buffer; 5 µl of 25 mmol/L MgCl$_2$; 200 mmol/L each of dATP, dCTP, dGTP, and dTTP; 1 µl of DMSO; 1 unit of Taq DNA polymerase; and 0.5 µl of 50 mmol/L PCR primers. The psoriasin primers were sense (5′-AAG AAA GAT GAG CAA CAC-3′) and antisense (5′-CCA GCA AGG ACA GAA ACT-3′) corresponding to the cDNA sequence,[13] or alternatively, PCR was performed with GAPDH primers, sense (5′-ACC CAC TCC TCC ACC TTT G-3′) and antisense (5′-CTC TTG TGC TCT TGC TGG G-3′).[23] For PCR amplification the reaction comprised an initial step of 5 minutes at 94°C, and then 45 cycles (30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C) for psoriasin or 35 cycles (45 seconds at 93°C, 45 seconds at 58°C, 30 seconds at 72°C) for GAPDH. PCR products of the two genes amplified from the same RT reaction were loaded into the same wells onto a 1.5% agarose gel before electrophoresis and ethidium bromide staining to visualize psoriasin (246 bp) and GAPDH (198 bp) cDNAs under UV illumination.

Preliminary experiments were performed with cell line and tumor RNA samples to establish the appropriate RNA input and PCR cycle number conditions to achieve amplification with both psoriasin and GAPDH primers in the linear range in a typical sample. Tumors from each cohort were processed as a batch, from frozen sectioning to RNA extraction, reverse transcription in triplicate, and then duplicate PCRs from each RT reaction. For each batch controls included RT-negative and RNA-negative controls and both psoriasin-positive (estradiol-stimulated MCF7) and psoriasin-negative (untransfected, wild-type MDA-MB-231 cells) RNA controls. All primary tumor PCR signals were assessed in gels and autoradiographs by video image capture and with a MCID-M4 image analysis program. Psoriasin expression was standardized to GAPDH expression assessed from the same RT reaction in separate PCR reactions and run in parallel on the same gel, and the mean of each duplicate PCR was then expressed relative to the levels in the MCF7 cell line standard. The invasive tumor component within each section was also assessed in the adjacent mirror image paraffin section, and the percentage area occupied by tumor was

used to correct for differences in epithelial cell content of the tumor sections used for RNA extraction.

Statistical Analysis

For analysis of associations, standardized psoriasin mRNA levels were used either as a continuous variable or transformed into low- or high-expression categories, using a level of one relative density unit. This cutpoint was selected to correspond to the lowest level at which protein could be detected by Western blot. Correlations with estrogen (ER) and progesterone (PR) receptor levels and inflammation were tested using Spearman's test. Associations with categorical variables were tested by either Mann-Whitney or analysis of variance tests for selected dependent variables, or unpaired *t-test for independent variables, or a χ^2 test.*

Results

Characterization of Psoriasin-Specific Antibody

Multiple S100 proteins are expressed in individual tissues and cells. To specifically distinguish psoriasin expression within archival formalin-fixed and paraffin-embedded tissues we raised a polyclonal antibody in chicken against a synthetic peptide that corresponded to the COOH terminus of psoriasin. This 14-amino acid region was selected on the basis of very low homology to other S100 proteins. Western blot analysis of an MDA-MB-231 breast cell line transfected with a plasmid incorporating psoriasin cDNA under the control of a CMV promotor (and known to express psoriasin mRNA by Northern blot; unpublished data) and breast tumors showed a single band corresponding to a protein of approx 11.7 kd with the chicken IgY antibody (Figure 1A). This signal could be completely inhibited by preincubation of the primary antibody with psoriasin synthetic peptide (data not shown) and was absent from the wild-type and vector-alone transfected MDA-MB-231 control cells. By comparison, a commercial anti-S100 antibody (Sigma), known to detect several S100 proteins in MDA-MB-231 cells,[24] weakly recognized the same 11.7-kd protein in transfected cells as well as several other S100 proteins in most samples (Figure 1B). Both antibodies reacted with additional higher molecular mass bands in tumor samples. However, specificity of the 11.7-kd psoriasin signal was further confirmed by Western blot using another anti-psoriasin polyclonal rabbit antibody previously raised against a recombinant psoriasin protein (data not shown).

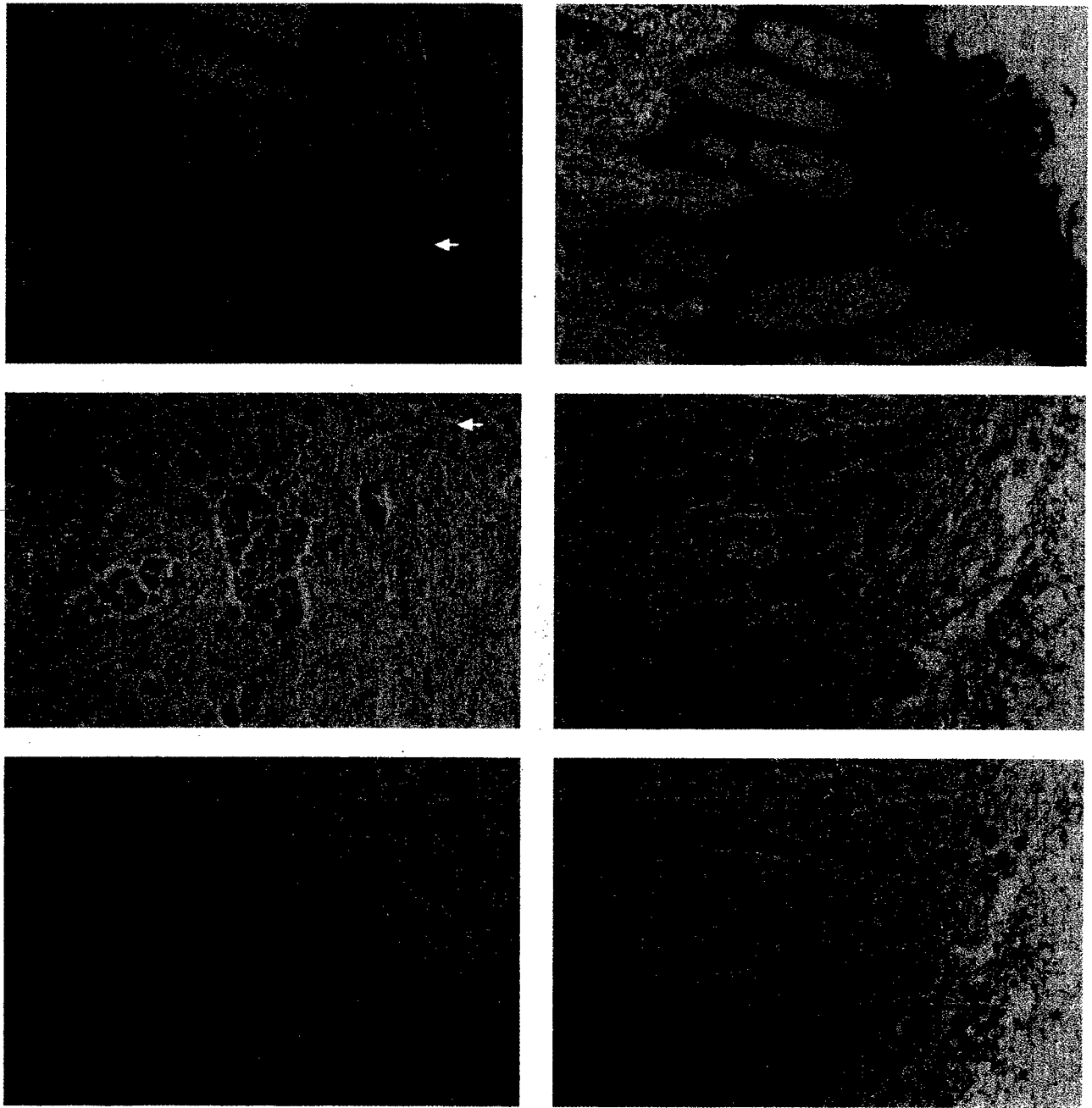### Localization of Cellular Expression of Psoriasin

To assess cellular localization of psoriasin we studied paraffin-embedded tissue blocks from breast, skin, and larynx by immunohistochemistry. The breast tumors studied possessed either high (six cases) or low (seven cases) levels of psoriasin mRNA and total protein expression (determined by Western blot and RT-PCR analysis of
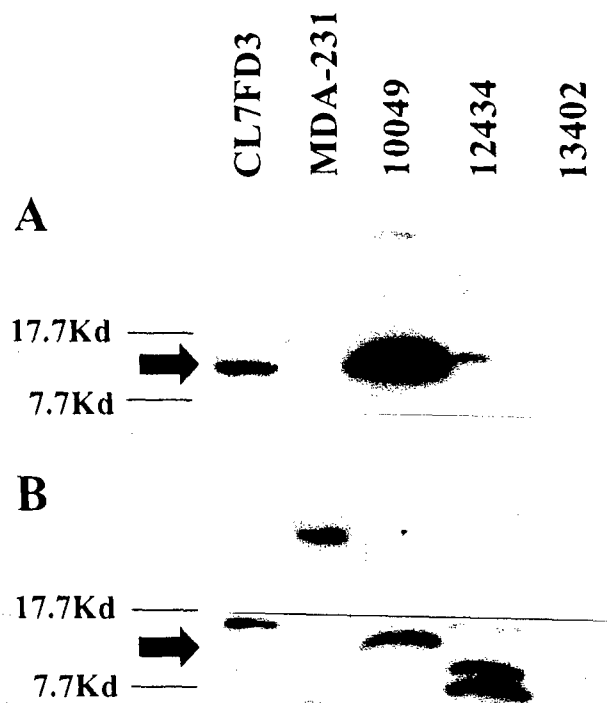
Figure 1. Western blot analysis of cell lines and tumors to demonstrate anti-psoriasin IgY antibody specificity. **A:** A protein band (approx 11.7 kd) detected using a chicken IgY anti-psoriasin antibody in a psoriasin-transfected MDA-MB-231 breast cell line and two tumors (10049, 12434), but absent in tumor 13402 and wild-type MDA-MB-231 cells. **B:** Detection of several S100-like proteins, using a commercial polyclonal S100 antibody applied to the same samples, in addition to weak detection of the same (approx 11.7 kd) protein band seen in **A**.

protein and RNA extracted from sections cut from the adjacent mirror-image frozen tissue blocks). Skin biopsies from the margins of two psoriatic lesions and a squamous carcinoma of larynx were also studied, as psoriasin was originally identified as a highly expressed protein in psoriatic skin and has also been identified as an expressed sequence tag in a cDNA library from laryngeal squamous carcinoma (http://www.ncbi.nlm.nih.gov/UniGene/Hs.112408). All cases were subjected to both immunohistochemistry and *in situ* hybridization on adjacent paraffin sections, and both signals were assessed independently, using a semiquantitative scoring system as described in Materials and Methods.

In breast tumors psoriasin protein was detected predominantly within epithelial tumor cells and was localized within both tumor cell nuclei as well as cytoplasm. Psoriasin was also present within some stromal cells and in some cases also on the luminal aspects of endothelial cells within small vessels (Figure 2). However, *in situ* hybridization demonstrated that mRNA expression was limited to epithelial tumor cells in all cases (Figure 2). The nuclear immunohistochemical staining was completely abolished by competition with the immunizing peptide and was not present in tumors that were negative for psoriasin but showed additional immunoreactive bands on Western blot (eg, see case 13402, Figure 1, and case 8840, Figure 4). Immunohistochemistry and Western blot were concordant in 12/13 cases. In one case Western blot analysis was negative and weak focal staining was

Figure 2. Immunohistochemical and *in situ* hybridization analysis of the cellular distribution and patterns of expression of psoriasin within psoriatic skin and breast carcinoma. Psoriasin protein is localized in hyperplastic epidermis of skin to both nuclei (**A**, **white arrow**) and cytoplasm (**A**, **black arrow**) of keratinocytes. Similar nuclear and cytoplasmic staining is seen in breast epithelial tumor cells (**C**, **black arrow**; case 8965). Psoriasin protein is also detected within occasional stromal inflammatory cells (**C**, **white arrow**). **E:** H&E-stained section from the same region of the tumor shown in **C**. Psoriasin mRNA expression in skin is restricted to epithelial cells in suprabasal layers of epidermis (**B**) and scattered invasive epithelial tumor cells in breast tumors (**D**), detected using antisense probe (**B** and **D**) compared to sense probe (**F**). Original magnification for all panels at the microscope, ×200.

seen by immunohistochemistry. Specificity of the nuclear signal was further confirmed by the fact that the presence of immunohistochemically detected protein expression, assessed on the basis of nuclear staining, was highly concordant (92%) with expression detected by *in situ* hybridization mRNA.

In skin, immunohistochemical staining was localized to keratinocytes within the mid to upper zones of the epidermis of skin showing psoriasiform hyperplasia. These keratinocytes corresponded to the cells that also showed mRNA expression by *in situ* hybridization in adjacent sections (Figure 2). The adjoining normal skin was negative. Occasional positive immunohistochemical staining, but no mRNA signal, was also observed in stromal cells in the dermis underlying the psoriatic lesion. As seen in breast tumor cells, psoriasin protein was localized both to the nucleus and cytoplasm within keratinocytes (Figure 2). The same nuclear and cytoplasmic localization was also detected in a squamous laryngeal carcinoma (data not shown). However, the polyclonal rabbit anti-psoriasin

antibody previously shown to provide immunofluorescent staining in frozen skin sections[13,25] did not detect any signal on paraffin sections from skin or breast. Additional experiments were performed with the chicken IgY anti-psoriasin antibody on skin and breast tumor sections in which immunohistochemical conditions (microwave *versus* protease antigen retrieval) and tissue treatment/fixation conditions (formalin *versus* alcohol *versus* paraformaldehyde *versus* frozen) were varied, and nuclear localization persisted under all conditions (data not shown).

Expression of Psoriasin mRNA in Invasive Breast Tumors

The changes in psoriasin expression previously observed in association with the transition from *in situ* to invasive carcinoma suggested a functional role in the early stages of progression. However, alteration of psoriasin expression in normal skin has also been associated with abnormal keratinocyte differentiation. To examine further the relationship of psoriasin with differentiation and invasiveness, we used RT-PCR and Western blot to examine psoriasin mRNA and protein levels in a cohort of invasive tumors. These tumors included several different tumor types and a range of differentiation, as determined by tumor grade and estrogen receptor status (Table 1).

Psoriasin mRNA was detected in all tumors by RT-PCR, but the levels varied considerably and were mostly low (Figure 3). Within the invasive ductal subgroup there was no significant difference in psoriasin expression with tumor grade. There was also no significant difference between tumor size or type, although there was a trend toward lower levels of expression in both well-differentiated tumor types, tubular and mucinous carcinomas, whereas lobular and medullary carcinomas showed a trend toward higher expression than invasive ductal tumors. However, higher levels of psoriasin mRNA expression showed a significant inverse correlation with both ER and PR levels ($r = -0.66$, $P = 0.0001$; $r = -0.47$, $P = 0.0003$, Spearman) and with ER and PR negative status (ER-ve *vs.* ER+ve; $n = 28$ *vs.* 29, mean (SD) 1.032 (0.7) *vs.* 0.32 (0.36), $P < 0.0001$ Mann-Whitney; PR-ve *vs.* PR+ve, $n = 25$ *vs.* 32, 1.05 (0.72) *vs.* 0.37 (0.40), $P < 0.0001$) in all tumors and within the invasive ductal subgroup. Psoriasin expression was also higher in axillary node-positive cases in all tumors (mean (SD) = 0.86 (0.73) *vs.* 0.59 (0.66), and the difference was statistically significant for the invasive ductal subgroup (mean (SD) = 0.88 (0.79) *vs.* 0.38 (0.28), $P = 0.035$, *t*-test). These relationships with ER, PR, and nodal status (Table 2) were also evident and remained statistically significant after correction of psoriasin levels for the relative tumor cell content, assessed as a percentage within the paraffin sections adjacent to the frozen tissue sections studied.

Psoriasin protein was detected by Western blot analysis in 10 tumors (Table 1 and Figure 4). These tumors (six ductal, two lobular, two medullary) corresponded to those with the highest mRNA levels observed by RT-PCR (above 1.0 arbitrary expression units). Also consistent

with RT-PCR analysis, Western blot-positive invasive ductal tumors were also significantly associated with ER-negative ($P < 0.0001$) and PR-negative ($P < 0.0012$) and node-positive ($P = 0.0143$) status (Table 2).

The relationship between psoriasin mRNA and protein expression and host inflammatory response was also examined (Table 2). Psoriasin mRNA showed a significant positive correlation in the entire cohort ($n = 57$, $r = 0.47$, $P = 0.0002$), in the entire cohort excluding the medullary carcinoma subgroup, which includes inflammatory infiltrates as a diagnostic criterion ($n = 52$, $r = 0.42$, $P = 0.0022$), and within the invasive ductal subgroup alone ($n = 34$, $r = 0.39$, $P = 0.023$). Cases with Western blot-detectable psoriasin protein also showed increased inflammatory infiltrates, both in the entire cohort (mean (SD) = 3.6 (1.1) *vs.* 2.3 (1.2), $P = 0.004$) and in the entire cohort excluding the medullary subgroup (mean (SD) = 3.3 (0.89) *vs.* 2.1 (0.98), $P = 0.007$).


Discussion

We have developed a psoriasin-specific antibody and confirmed its specificity as well as its ability to detect the psoriasin protein in formalin-fixed and paraffin-embedded specimens. We have shown that there is a high concordance between psoriasin mRNA and protein levels in invasive tumors, and persistance of psoriasin expression at higher levels is significantly associated with poor prognostic markers, including ER- and PR-negative and lymph node-positive status. Psoriasin expression within breast tumor cells is also associated with inflammatory infiltrates.

Indirect support for a role for S100 genes in breast tumor progression is provided by several observations. Disruption of calcium signaling pathways has been implicated as a central mechanism in tumorigenesis and specifically in the process of invasion and metastasis.[26] Moreover, the chromosomal location of the S100 gene family lies in a region of chromosome 1 that frequently (>50%) shows loss of heterozygosity in invasive tumors.[27] Furthermore, several S100 genes are expressed in breast cell lines and tumors and are known to manifest alteration of their expression in association with tumorigenesis and progression.[11,24] In particular, S100A2 and S100A4 have been identified to be differentially expressed between normal and neoplastic cells[3,28,29] and up-regulated in metastatic as compared to nonmetastatic cells in both mouse and rat mammary tumor cell lines.[5,30] *In vivo* studies of breast tumors have also shown a correlation between high levels of S100A4 expression, nodal metastasis, and ER-negative status.[31] More direct evidence has emerged from modulation of S100A4 expression in transfected cell lines that have shown that over-expression of S100A4 can also induce the metastatic phenotype in mouse, rat, and human cells.[4,6,32] Furthermore, there is evidence that S100A4 may exert its effect on cell cytoskeleton[8,9] and motility,[7] and it has also been demonstrated that up-regulation of S100A4 in mouse tumor cell lines can down-regulate expression of E-

Table 1. Clinicopathological Parameters, Histological Composition of the Tumor Section, and Psoriasin Expression in 57 Invasive Breast Carcinomas Assessed by RT-PCR and Western Blot

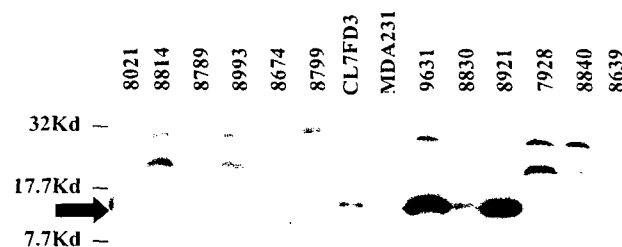| TB# | Clinicopathological parameters | | | | | | | Psoriasin | | |
	Type	ER	PR	GrSc	Size	NS	Inf	RT-PCR	RT-PCR/Inv%	WB
11549	muc	194	133		3	−	2	0.06	0.15	−
10515	muc	341	176		3	−	1	0.08	0.14	−
9948	muc	46	22		6.5	−	1	0.10	0.16	−
10582	muc	109	62		2.3	na	1	0.14	0.34	−
8832	muc	295	177		4	−	2	1.94	2.77	−
8021	muc	331	328		2.3	−	2	0.11	0.15	−
11387	tub	105	35		3.5	na	2	0.09	0.29	−
9483	tub	56	0		1.2	−	2	0.09	0.91	−
11651	tub	67	24		2.2	−	3	0.23	0.77	−
8814	tub	232	103		2	−	2	0.44	1.45	−
8720	tub	29	73		2	−	1	0.52	5.21	−
12072	tub	8.3	5		2.3	+	3	0.67	1.34	−
13041	med	3.4	9		2	−	5	0.40	0.49	−
13153	med	4.9	2.4		3	na	5	0.61	0.76	−
11867	med	1.4	9		1.6	+	5	1.60	2.67	+
13058	med	4.6	12		2.8	−	5	1.63	2.04	−
12434	med	1	1.3		1.2	−	5	1.63	3.27	+
8639	ilc	52	83		na	−	1	0.20	0.67	−
8799	ilc	111	139		6	+	2	0.31	3.15	−
8993	ilc	142	528		8	+	1	0.52	0.86	−
9801	ilc	2.1	9.8		na	−	3	0.56	1.60	−
8921	ilc	2.3	8.9		8	−	2	2.07	3.77	+
8961	ilc	0.7	3.4		2.5	−	3	2.34	5.84	+
9000	idc	392	596	7	2.5	−	1	0.07	0.09	−
13402	idc	49	35	4	2.8	−	2	0.07	0.17	−
11971	idc	97	25	4	1.5	−	2	0.13	0.42	−
8684	idc	74	43	7	5	+	1	0.14	0.35	−
12853	idc	17.3	83	9	4.8	+	4	0.15	0.22	−
8840	idc	74	68	7	1.8	+	3	0.17	0.37	−
8834	idc	10	147	5	2	−	2	0.17	0.34	−
8674	idc	16.7	4.5	9	na	−	2	0.19	0.35	−
12037	idc	225	144	4	3.5	+	2	0.20	0.40	−
12868	idc	93	141	9	3.5	na	1	0.21	0.28	−
8599	idc	58	81	4	3.5	−	1	0.24	0.79	−
10105	idc	0.9	3.8	9	3	+	4	0.24	0.40	−
7928	idc	33	72	5	3	+	2	0.27	0.67	−
13414	idc	15.5	59	5	4.1	−	2	0.28	0.56	−
11343	idc	78	44	4	na	−	3	0.29	0.73	−
10644	idc	130	4.7	9	3.2	+	2	0.32	0.81	−
10137	idc	42	26	7	1.8	−	1	0.44	0.89	−
10064	idc	0.8	4.6	9	2.5	na	2	0.53	0.88	−
11769	idc	1.1	3.5	7	na	−	3	0.56	0.80	−
8932	idc	114	27	4	2	−	1	0.56	1.13	−
10906	idc	46	6.6	9	4.5	na	5	0.58	0.64	−
8789	idc	0.8	0.4	7	na	na	3	0.66	1.64	−
10150	idc	70	42	7	na	−	1	0.67	1.68	−
11459	idc	3.6	98	5	4.6	+	3	0.67	0.96	−
13191	idc	17.2	9.2	9	3.2	−	2	0.69	0.87	−
10124	idc	1.9	12.9	9	3	−	4	1.00	1.42	−
8830	idc	0.7	8	9	6	+	4	1.06	1.32	+
8790	idc	6	50	5	1.5	+	2	1.07	3.58	−
11118	idc	6.6	11.8	5	8.5	+	2	1.10	2.20	−
12715	idc	1.5	16	7	3	na	3	1.24	2.06	+
9631	idc	0.7	4.5	9	na	+	4	1.32	3.10	+
8965	idc	0.4	9.9	7	na	+	4	1.85	2.64	+
10049	idc	0.8	14	9	3.7	+	4	2.01	5.04	+
8704	idc	0.7	3.5	7	3.5	+	2	2.60	6.50	+

TB, tumor bank case number; type, mucinous (muc), tubular (tub), medullary (med), lobular (ilc), ductal (idc); ER, PR, estrogen/progesterone receptor levels (fmol/mg protein); GrSc, Nottingham grade score; Size, tumor size (cms); NS, nodal status, positive (+), negative (−), not available (na); Inf, estimate of inflammatory infiltrate, low (1) to high (5). RT-PCR, psoriasin mRNA level determined by RT-PCR; RT-PCR/Inv%, psoriasin mRNA level determined by RT-PCR and adjusted for the percentage tumor cell content of the tissue section (as described in Materials and Methods); WB, psoriasin protein level determined by Western blot.
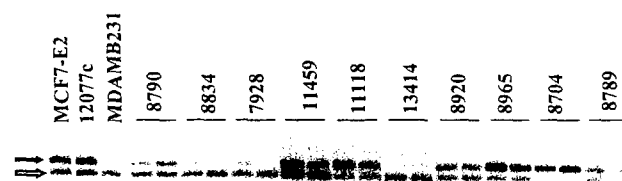
Figure 3. RT-PCR analysis of psoriasin mRNA expression in invasive breast tumors. Psoriasin (**upper black arrow**) and GAPDH (**lower open arrow**) from duplicate PCRs of 10 representative tumors. Control lanes include estradiol-treated MCF7-E2 cells, a tumor control 12077c, and wild-type MDA-MB-231 cells.

Figure 4. Western blot analysis of psoriasin protein expression in invasive breast tumors. Psoriasin (**black arrow**) is detected in 3/12 representative tumors and within the positive control (CL7FD3).

cadherin and disturb the intracellular distribution of B-catenin.[16]

A possible role for psoriasin (S100A7) in breast cancer first emerged when it was also identified as a cDNA down-regulated in a nodal metastasis relative to a primary breast tumor.[33] Nevertheless, the significance of the initial observation was unclear because of the fact that expression was only detectable in a small proportion of cells within invasive primary tumors studied by in situ hybridization and overall could be detected in only 18% of primary tumor specimens assessed by Northern analysis. An explanation for this paradox became apparent when psoriasin was also identified by us as a gene that is particularly highly expressed in the ductal epithelial cells of preinvasive ductal carcinoma in situ,[1] which can be present as a significant component with invasive tumor specimens. We have now shown that when higher levels of psoriasin expression persist within invasive tumors, this correlates with indicators of increased metastatic potential. It should be noted that the strong relationship with ER status is compatible with studies of S100A4[31] and the in vitro observation[33] (and our unpublished data) that psoriasin is regulated by estradiol in MCF7 cells. Although it is interesting that the nature of this correlation is different between the in vitro and in vivo situations,

similar differences have been found with other genes in breast tumors,[34] suggesting that additional external factors may influence psoriasin regulation in vivo.

Although the biological effect of alteration of psoriasin in breast tumors is currently unknown, it is interesting to speculate from this pattern of expression that psoriasin may be important in the invasive phenotype.[16] This role might be mediated through an indirect influence on the effector cells of the host immune response or perhaps through a more direct influence on the epithelial tumor cell. The first hypothesis is supported by the correlation seen here with the degree of host inflammatory cell response within breast tumors and the previous evidence that implicates psoriasin as a chemotactic factor.[14] However, psoriasin protein was only detected in approximately 50% of medullary and ductal tumors with marked inflammatory responses. The second hypothesis is supported by our observation that psoriasin may not only be secreted[13,15] but also can be localized in both nuclear and cytoplasmic compartments in normal skin and breast tumors. Although further studies beyond immunohistochemistry are necessary to confirm this observation, the pattern of expression is consistent between cells in two

Table 2. Relationship between Psoriasin Expression and Prognostic and Tissue Factors

		All				IDC			
		n	Low Ps	High Ps		n	Low Ps	High Ps	
ER	−	28	14	4	P = 0.0001	19	10	9	P = 0.0019
	+	29	28	1		15	15	0	
PR	−	25	13	12	P = 0.001	15	8	7	P = 0.018
	+	32	29	3		19	17	2	
NS	−	30	24	6	ns (P = 0.095)	14	13	1	P = 0.0002
	+	19	11	8		15	8	7	
INFL	Low	34	29	5	P = 0.049	20	17	3	ns (P = 0.07)
	High	18	11	7		14	8	6	
Size	<2	12	9	3		6	5	1	
	2–5	29	22	7	ns	18	14	4	ns
	≥5	7	4	3		3	1	2	
Grade	Low					12	10	2	
	Mod					10	7	3	ns
	High					12	8	4	
Type	idc	34	25	9					
	ilc	6	4	2					
	med	5	2	3	ns				
	muc	6	5	1					
	tub	6	6	0					

ER, PR, estrogen/progesterone receptor status; NS, nodal status; INFL, inflammatory infiltrate; Size, tumor size (cms); Grade, Nottingham grade; Type, mucinous (muc), tubular (tub), medullary (med), lobular (ilc), ductal (idc); Low Ps/High Ps, low/high psoriasin mRNA level determined by RT-PCR (cutpoint values used as described in Materials and Methods). P values determined by χ^2 or ANOVA tests. ns, not significant.

closely related epithelia, epidermis and breast ductal epithelium, and the detection of nuclear and cytoplasmic signal was unrelated to tissue fixation or immunohistochemistry protocol, which may effect staining with some antibodies.[35,36] Dual localization and alteration of the subcellular localization with disease has also been observed with another S100 related keratinocyte protein, profilaggrin, expressed in the epidermis.[2,37] Similarly, altered cellular distribution of proteins such as BRCA1 and B-catenin are also recognized to be an important aspect of tumor progression.[38–40] Furthermore, other S100 proteins have previously been associated with both extra- cellular and intracellular actions,[41] and previous studies have also indicated potential interactions for S100A4 with both cytoskeletal[8,9] and nuclear[42] proteins. It has also recently been shown that other secreted S100 proteins can be localized to cytoplasm and nucleus,[43,44] and specifically S100A2 has been found in breast cell nuclei, whereas S100A6 localizes to the cytoplasm[24]; however, the functional significance of these findings remains unknown.

In conclusion, we have shown that expression of psoriasin (S100A7) mRNA and protein correlates with indicators of poor prognosis in invasive breast tumors, including ER, PR, and nodal status, but is not related to differentiation, as manifested by invasive tumor type or grade. The relationship observed between psoriasin and the inflammatory response is also compatible with a role as a chemotactic factor; however, the possibility of additional intracellular functions is raised by the presence of its nuclear localization in both skin and breast tumors. Further studies will be necessary to confirm the latter observation and pursue the biological functions of psoriasin in relation to breast tumor progression.

Acknowledgments

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Original Paper

Lumican and decorin are differentially expressed in human breast carcinoma

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Abstract

Previous studies have shown that lumican is expressed and increased in the stroma of breast tumours. Lumican expression has now been examined relative to other members of the small leucine-rich proteoglycan gene family in normal and neoplastic breast tissues, to begin to determine its role in breast tumour progression. Western blot study showed that lumican protein is highly abundant relative to decorin, while biglycan and fibromodulin are only detected occasionally in breast tissues ($n = 15$ cases). Further analysis of lumican and decorin expression performed in matched normal and tumour tissues by *in situ* hybridization showed that both mRNAs were expressed by similar fibroblast-like cells adjacent to epithelium. However, lumican mRNA expression was significantly increased in tumours ($n = 34$, $p < 0.0001$), while decorin mRNA was decreased ($p = 0.0002$) in neoplastic relative to adjacent normal stroma. This was accompanied by a significant increase in lumican protein ($n = 12$, $p = 0.0122$), but not decorin. Further evidence of altered lumican expression in breast cancer was manifested by discordance between lumican mRNA and protein localization in some regions of tumours but not in adjacent morphologically normal tissues. It is concluded that lumican is the most abundant of these proteoglycans in breast tumours and that lumican and decorin are inversely regulated in association with breast tumourigenesis. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: lumican; decorin; small leucine-rich proteoglycan; breast cancer; tumour progression

Introduction

The development and progression of breast carcinoma are caused by alterations in the expression of multiple genes, most of which are responsible for normal physiological pathways and the necessary cellular interactions to support these functions within the mammary gland. These include alterations in the interactions between the epithelial and stromal cells, which are manifested in tumours by well-recognized morphological changes known as the stromal reaction [1]. Such alterations in stromal–epithelial interactions may influence the risk of transformation of the breast epithelial cell and may contribute very early steps in tumourigenesis, as has recently been proposed in other systems [2]. However, the net effect of these alterations in the stroma on the later stages of tumour progression is unresolved [3].

Resolution of this issue is complicated by the recognition that the stroma is a highly complex tissue that includes a variety of different types of fibroblasts [4] and a range of proteins, glycoproteins, and proteoglycans which may play a role in tumour biology. We have recently extended this list by identifying lumican, a member of the small leucine-rich proteoglycans (SLRPs) as an mRNA that is expressed in the stroma of normal breast tissues and is overexpressed in invasive carcinomas [5]. Members of this family of proteoglycans have been implicated principally in matrix assembly and structure [6], but also more recently in the control of cell growth [7]. While studies of decorin have shown altered expression in neoplastic stroma [3], lumican has previously been studied only in the context of connective tissue and corneal disease [8,9], and the role of SLRPs in human breast cancer is relatively unexplored. To explore further the potential role of lumican and related genes in breast tumour progression, we have now examined the expression of lumican relative to that of other members of the SLRP family, decorin, biglycan and fibromodulin, at both mRNA and protein level, in normal and neoplastic breast tissues.

Materials and methods

Human breast tissues

All breast tumour cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As has been previously described [10], tissues are accrued to the bank from cases at multiple centres within Manitoba, rapidly collected, and processed to create matched formalin-fixed, paraffin-embedded, and frozen tissue

blocks with the mirror image surfaces orientated by coloured inks. The histology and cellular composition of every sample in the bank are interpreted in haematoxylin and eosin (H&E)-stained sections from the face of the former tissue block.

For the initial study to compare broadly the expression of different members of the SLRP gene family, a mixed pilot cohort was selected from the Tumor Bank to include nine different invasive carcinomas, three normal tissue samples from patients with cancer, and three normal tissues from normal patients without cancer. The invasive tumours included different tumour types (five ductal, three lobular, and one tubular carcinoma), grades (four high, one moderate, four low Nottingham grades), and oestrogen receptor (ER) levels (three ER <10 fmol/mg, three ER 10–20, three ER 39–169), and total stromal fractions ranging from 50 to 95% of the cross-sectional area. The mean patient ages were 62, 70, and 28 years for each subgroup, respectively (tumour tissues, normal tissues adjacent to tumours, and normal tissues).

For the subsequent studies to compare lumican and decorin expression, a second more defined and homogeneous cohort of 46 cases was selected to provide matching primary tumour tissues and adjacent normal tissue. This cohort included only invasive ductal carcinomas and was primarily selected to ensure availability of histologically confirmed and distinct regions comprising morphologically normal and tumour tissue elements in different blocks (12 cases, for western blot studies) or the same block (34 cases, for *in situ* hybridization studies). The subset used for western blot studies was also selected to possess equivalent cross-sectional areas [mean section area$^{(SD)}$ in tumour tissues $=0.86^{(0.44)}$ cm^2, adjacent normal tissues $=0.85^{(0.35)}$ cm^2] and stromal content [mean stromal area$^{(SD)}$ in tumour tissues $=68^{(10)}$%, adjacent normal tissues $=89^{(6)}$%] between the matching blocks and to incorporate cancer cases from both post-menopausal (six cases mean$^{(SD)}$ $=76^{(7)}$ years) and pre-menopausal patients (six cases mean$^{(SD)}$ $=44^{(3)}$ years).

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotting

Total proteins were extracted from frozen tissue sections. These were cut from the face of frozen tissue blocks immediately adjacent to the face of a matching paraffin block [11] from which paraffin sections had been previously cut for pathological assessment and for *in situ* hybridization. For the first cohort of cases, an average of 20 20 μm tissue sections were cut from each typical tissue block (0.5 × 1.0 cm^2 cross-sectional area) and used for extraction; however, the number of tissue sections was varied for each case according to the measured area of the tissue within individual blocks, to ensure that equivalent volumes of tissue were used for the extraction, which was done as described previously [12]. For the second cohort of matching tissue samples, the same number of frozen

sections (20 × 20 μm) was cut from the measured surface of each tissue block together with a single section from the adjacent paraffin block. This was used as a reference for composition and protein extraction was then performed on the frozen sections with equivalent volumes of extraction buffer. Proteins present in equivalent volumes of extracts were analysed by SDS/PAGE and immunoblotting, using anti-peptide antibodies specific for the carboxyl-terminal regions of the core proteins of lumican, decorin, fibromodulin, and biglycan [12–14]. The specificity of all antibodies was verified by peptide absorption and SLRP cross-reactivity analysis. Protein signals were detected by chemiluminescence and photographed prior to quantitation by video-image analysis and densitometry using an MCID M4 system and software (Imaging Research, St Catherines, Ontario, Canada). All signals were then adjusted with reference to control cartilage samples run with each blot. For the second cohort of matched tissue samples, signals were also adjusted with reference to the measured cross-sectional area and the stromal content of the tissue block to control for equivalent loading. Additional analysis was performed on all signals after further adjustment for relative stromal content of the tissue sections assessed in adjacent H&E sections.

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections using the same antibody to lumican as used for immunoblotting [9,12]. Sections (5 μm thick) were obtained from paraffin-embedded tissue blocks matching the frozen tissue blocks of those cases used for reverse transcription-polymerase chain (RT-PCR) and protein analysis. After deparaffinizing, clearing, and hydrating in TBS buffer (Tris buffered saline, pH 7.6) the sections were pretreated with 3% hydrogen peroxide for 10 min to remove endogenous peroxidases and non-specific binding was blocked with normal swine serum, 1:10 (Vector Laboratories S-4000). TBS was used between steps to rinse and as a diluent. Primary antibody to lumican was applied at a 1:400 dilution overnight at 4°C, followed by biotinylated secondary swine anti-rabbit IgG, 1:200 (DAKO) for 1 h at room temperature. Tissue sections were incubated 45 min at room temperature with an avidin/biotin horseradish peroxidase system (Vectastain ABC Elite, Vector Lab.) followed by detection with DAB (diaminobenzidine), counterstaining with 2% methyl green, and mounting. A positive tissue control (colonic mucosa) and a negative reagent control (no primary antibody) were run in parallel. Immunostaining patterns and intensity were assessed by light microscopic visualization.

In situ hybridization

Paraffin-embedded 5 μm sections of breast tissues were analysed by *in situ* hybridization according to a previously described protocol [5]. For lumican, the

plasmid Lumi-398, which consisted of pGEM-T plasmid (Pharmacia Biotech), containing a 398 bp portion of lumican cDNA between bases 1332 and 1729, was used as a template to generate UTP^{S35} labelled sense and antisense riboprobes using Riboprobe Systems (Promega, Madison, WI, USA) and either the T7 or SP6 promotor at the 5′ or 3′ end of the lumican sequence according to the manufacturer's instructions. For decorin, the plasmid Dec-322 was used as a template. This consisted of pGEM-T plasmid containing a decorin insert with a comparable length (322 bp) to the lumican probe generated by PCR amplification from the decorin cDNA [12] using primers that corresponded to decorin (sense 5′-AAATGCCCAAAACTCTTCAG-3′ and antisense 5′-AAACTCAATCCCAACTTAGCC-3′) [15]. All PCR cDNAs and plasmid inserts were sequenced to confirm their entity. Levels of lumican and decorin expression were assessed in normal and tumour regions by microscopic examination at low magnification and with reference to the negative sense and positive control tumour sections. This was done as previously described [5] by scoring the estimated average signal intensity (on a scale of 0–3) and the proportion of stromal cells showing a positive signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0, greater than one-half). The intensity and proportion scores were then multiplied to give an overall score. Regions with a score lower than 1.0 were deemed negative or weakly positive.

Microdissection and protein extraction analysis

To assess protein localization within regions of tumours, two cases were selected that showed marked and well-defined regions within the same tissue section with discrepancies between mRNA and protein expression. This was determined by *in situ* hybridization and immunohistochemistry in adjacent serial sections from paraffin tissue blocks. The mirror image frozen tissue blocks to these paraffin blocks were used for microdissection as previously described [11] and protein was extracted from these histologically defined regions as described above. Briefly, thin 5 μm frozen sections were cut from the faces of the frozen tissue blocks and stained by H&E, and the relevant histological regions of approximately 1–2 mm^2 distinguished and confirmed by reference to the paraffin sections already studied. Multiple thick frozen sections (20 × 20 μm) were then cut, rapidly stained, and microdissected at room temperature from each section in turn, and the microdissected tissue fragments were frozen again prior to protein extraction.

Results

Identification of lumican as the most abundant SLRP in normal and neoplastic breast tissues

To determine the relative importance of altered lumican expression in breast tumourigenesis, the expression of lumican protein was compared with that

of three other members of the SLRP family, decorin, fibromodulin and biglycan, by western blot in a heterogeneous panel of nine breast tumours and six normal tissues.

Lumican was highly abundant in all samples and in both neoplastic and normal tissues (Figure 1). A significant increase was seen in the mean level of lumican protein between normal and tumour [mean$^{(SD)}$ tissue adjusted optical density units, normal = 0.43$^{(0.08)}$, tumour = 0.56$^{(0.15)}$, $p = 0.026$, Mann–Whitney test]. Although an apparent difference in the level of lumican between normal samples from normal patients and normal samples adjacent to tumours was seen, this difference did not persist when the different stromal content of these samples was taken into account. Similarly, there was no difference in the levels in tumour tissues on comparing pre- and post-menopausal patients. Nevertheless, an increase in the overall molecular weight and polydiversity was noted between normal tissues and morphologically normal tissue adjacent to tumours, which might be attributable to either different age or association with tumour in the adjacent breast.

In comparison, decorin, although also present in most samples examined by western blot, was much less
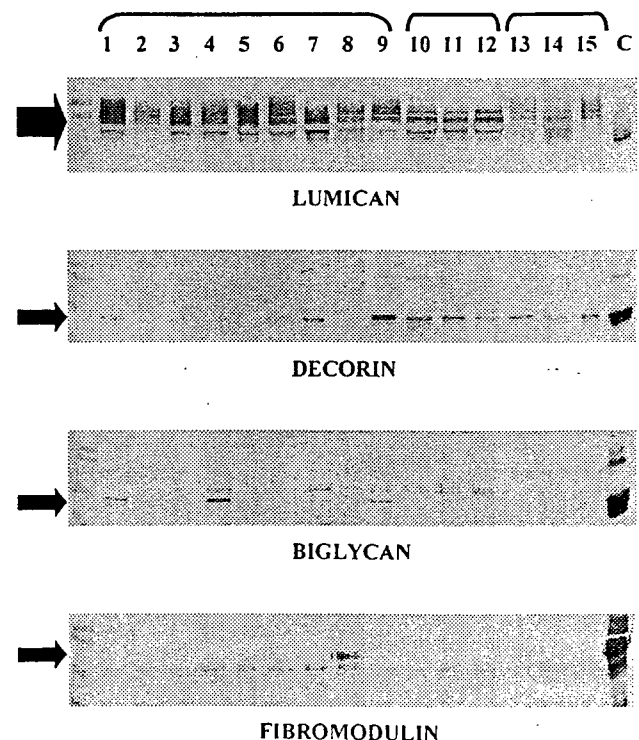
Figure 1. Immunoblotting study of lumican, decorin, biglycan, and fibromodulin protein expression in human breast tumours (lanes 1–9); normal tissues from normal patients (lanes 10–12); and normal tissues adjacent to carcinomas (lanes 13–15). All protein samples were extracted from sets of frozen tissue sections bracketed by sections assessed by H&E stain and light microscopy to confirm content. Chemiluminescent signals for decorin, biglycan, and fibromodulin required three-fold longer exposure times than that for lumican. Molecular markers (left) and cartilage control sample (right) are present in all panels

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abundant relative to the cartilage control (Figure 1). It should be noted that the decorin (in common with biglycan and fibromodulin) signals shown in Figure 1 also required a three-fold longer chemiluminescent exposure time (9 s) than that for lumican (3 s). However, in contrast to lumican, there was a marked decrease in decorin between normal and tumour samples [mean[(SD)] optical density units; normal = $0.21^{(0.06)}$, tumour $= 0.13^{(0.14)}$, $p = 0.066$, Mann–Whitney test]. No difference was seen in the signals between normal samples from normal and cancer patients.
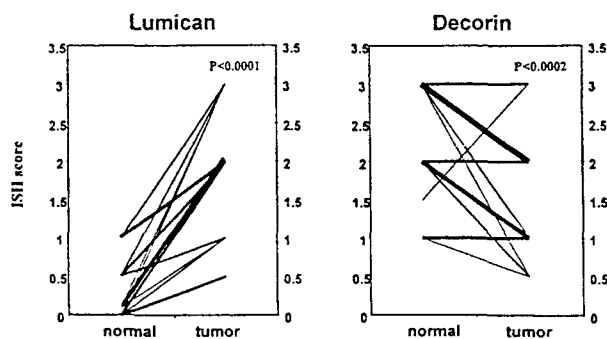
Fibromodulin expression was not detected in normal tissues and at only low levels in only 3/9 tumours, where the presence of fibromodulin correlated with those tumours with the highest content of epithelial tumour cells. Biglycan was also only detected at low levels in 2/6 normal tissues and 3/9 tumours, where in contrast to fibromodulin, its presence correlated directly with those tumours with the highest content of collagenous stroma.

Lumican and decorin are differentially expressed between normal and neoplastic tissues
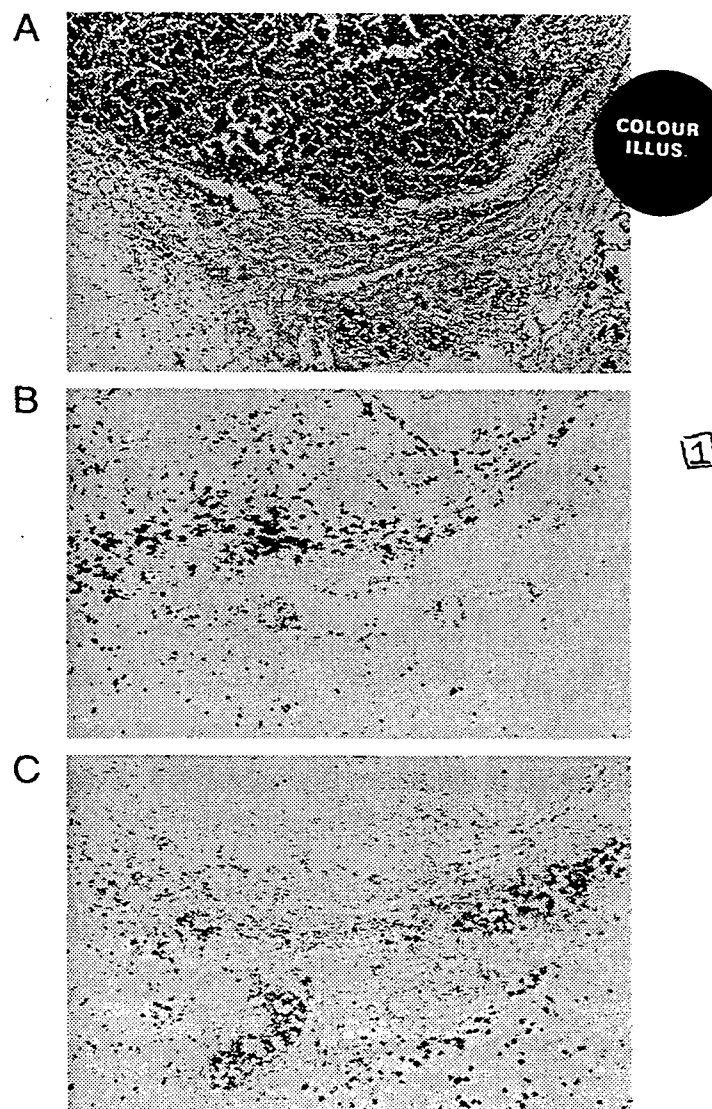
In order to examine further the distinct alterations in the expression of lumican and decorin, the mRNA and protein expression of both genes was examined in 46 cases by in situ hybridization (34 cases) and western blot (12 cases) from the second cohort of cases, comprising matched normal and tumour samples.

As previously shown, prominent lumican mRNA expression was detected, using an antisense probe, in stromal fibroblast-like cells within the tumour and immediately adjacent to invasive tumour cells. Assessment of mRNA levels using a semi-quantitative approach, as detailed in the Materials and methods section, also confirmed our previous observations [5] made on a different set of tumours, and lumican mRNA was found to be significantly elevated in the majority of tumours when levels were compared with those present in adjacent normal stroma ($p < 0.0001$, Wilcoxon test, Figures 2 and 3B). Higher levels of

lumican (≥ 1) were present in tumour than in normal tissue in 26/34 cases. At the same time, decorin levels also showed a consistent and significant difference, with lower levels seen in stroma associated with tumour, relative to stroma associated with adjacent normal tissue components ($p < 0.0002$, Wilcoxon test, Figures 2 and 3C), with lower levels of decorin (≥ 1) present in tumour than in normal tissue in 22/34 cases. The pattern of expression of decorin was also identical in sections from the same cases studied with a different in situ hybridization riboprobe (data not shown). Although we have previously noted a relationship between lumican and poor prognostic factors, these associations were not found in the present larger series.

Figure 3. Lumican and decorin mRNA expression detected by in situ hybridization within a breast tumour section. Panel A (H&E section) shows the histology including the invasive tumour (upper area), the tumour margin (middle), and adjacent normal tissue including lobular-ductal units (lower area). Lumican expression (B) is high within the tumour and tumour margin and lower in the normal fat and collagenous stroma adjacent to the normal lobules. Decorin (C) shows high expression in the normal stroma adjacent to normal lobules and reduced expression in the tumour. ×340

Figure 2. Lumican and decorin mRNA levels in matched normal and tumour tissues, assessed by in situ hybridization and semi-quantitative scoring as described in the Materials and methods section. The thickness of each line (on a scale of 1–9) corresponds to the number of cases showing the same differences in scores (n = 34 cases)

In keeping with the pattern of mRNA expression, the mean lumican protein signal assessed by western blot was also higher in 9/12 tumours relative to normal tissues [mean$^{(SD)}$ optical density units, normal = 0.22$^{(0.15)}$, tumour = 0.43$^{(0.19)}$, p = 0.0122 Wilcoxon test]. Once again, in contrast to this, decorin protein was lower in 7/12 tumours relative to normal tissues, but in this case the differences were not statistically significant [mean$^{(SD)}$ optical density units, normal = 0.22$^{(0.19)}$, tumour = 0.17$^{(0.2)}$, p = ns (not significant), Wilcoxon test]. These contrasting patterns of lumican and decorin expression also persisted after standardization of western blot signals for relative stromal content (data not shown).

Lumican mRNA and protein expression can occur in different regions within breast tumours

Immunohistochemical study of the lumican distribution within the same tissues that had already been examined by *in situ* hybridization was performed using the same antibody [9,12] that had been employed for western blot analysis (Figure 4). This showed that lumican was abundant throughout the collagenous stroma of both normal and tumour sections, with prominent deposition around small vessels, breast duct, and lobular structures. There was increased deposition within the collagenous stroma of tumours, in particular at the invasive margins and in areas of dense collagen within central regions of some tumours, compared with adjacent normal tissues. However, in some cases there were distinct regions, up to 2 mm in area within the tumour sections, containing loose stroma in which there was a complete absence of lumican detectable by immunohistochemistry (Figures 4C and 4D); but the same regions showed high expression when examined for lumican mRNA by *in situ* hybridization in adjacent sections (Figures 4A and 4B). Similarly, other areas showed strong staining for lumican protein, but low levels of mRNA.

To explore the possibility that the absence of lumican expression detected by immunohistochemistry might be due to the conformation of the native protein or the binding of lumican to other proteins, resulting in the masking of the carboxy-terminal epitope, specific areas measuring approximately 1 mm^2 each were microdissected from frozen sections of two tumours and lumican protein was assessed under denaturing conditions by SDS/PAGE and western blot. In both cases, those regions with high mRNA expression and negative by immunohistochemistry were also negative by western blot, while areas showing very low mRNA expression but strong staining by immunohistochemistry were positive by western blot (Figure 5).

Discussion

We have shown that lumican is the most abundant proteoglycan in comparison with several other members of the family of small leucine-rich proteoglycans (SLRPs) in breast cancer. We have also extended our previous observations [5], based on the detection of lumican mRNA, in showing that the total lumican protein is also increased in breast tumours relative to adjacent normal tissues. Our results also demonstrate that this pattern of up-regulation of lumican in relation to breast tumourigenesis is distinct from that of the closely related decorin gene, which is inversely regulated and reduced at mRNA and to a lesser extent at protein levels, in tumour relative to adjacent normal tissue. Finally, we have shown that lumican expression in tumours may also be associated with an abnormal distribution within the stroma, manifested by discordance between mRNA and protein deposition within subregions of breast tumours.

The family of SLRPs share several common features, including a central region of leucine-rich repeats bounded by flanking cysteine residues, and localization in the extracellular matrix. The SLRPs can be separated into three subgroups that include decorin and biglycan, lumican and fibromodulin, and epiphycan and osteoglycin, which are distinguishable by amino acid homologies and also by gene structure [16]. Decorin, probably the best studied of these genes, is known to interact with a variety of extracellular matrix molecules and has been shown to be capable of influencing collagen fibril growth and assembly both *in vitro* and *in vivo* [6,7]. Decorin may also influence tumour cell growth through indirect effects on the availability of growth factors from the extracellular matrix, or directly through activation of the EGF receptor and induction of the p21 cell-cycle inhibitor [18]. In contrast, less is known about lumican and other SLRPs. However, *in vitro* and *in vivo* data indicate that lumican is also important in the regulation of collagen fibril assembly [19]. This view is supported by recent observations based on mice with homozygous deletion of the lumican gene, where loss of corneal transparency and increased skin fragility are associated with disorganized and loosely packed collagen fibres related to increased and irregular fibril size, and interfibrillar spacing, as viewed by light and electron microscopy [8].

The observation that lumican is highly abundant compared with other SLRPs in breast tumours cannot be interpreted to mean that it is necessarily the most important. This is underscored by the recent demonstration that although decorin is apparently more abundant than versican in prostate cancer tissue, only an increase in the larger chondroitin sulphate proteoglycan versican correlates with grade, and inversely with progression-free survival, in prostate cancer [20]. Similarly, the increase in lumican as seen here in association with breast tumourigenesis may be less important than the parallel decrease in decorin. It should also be noted that while the present study was focused primarily on examining the relative expression of SLRPs between matched normal and tumour tissues and was not necessarily designed to compare levels between cases, we did not observe any significant

A

B

COLOUR
ILLUS.

C

D
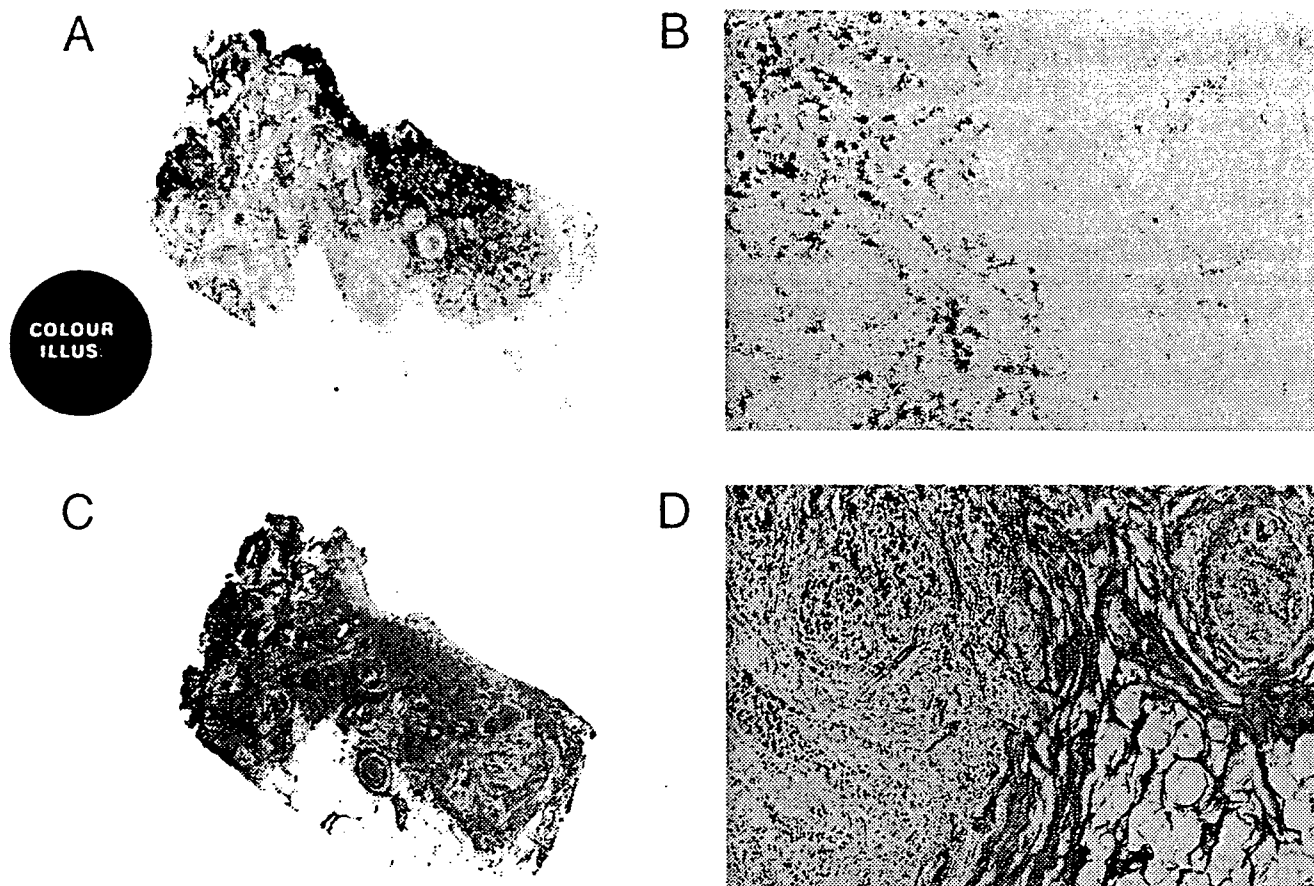
Figure 4. *In situ* hybridization and immunohistochemical study showing regional discordance in lumican mRNA (A, B) and protein expression (C, D) displayed in adjacent sections in breast tumours. Panels A and C show the overall pattern of mRNA (A, black signal) and protein (C, brown staining) within a tissue section (0.4 × 0.8 cm in size) that includes regions of *in situ* and invasive tumour (upper left and upper middle) and adjacent normal tissue (lower left and lower right). Panels B and D show a detailed microscopic view (× 400) of the cellular localization of mRNA and protein within a small region at the invasive edge within the same section (tumour component at left, normal component at right)

relationship between lumican or decorin and prognostic factors within this larger tumour cohort, as previously noted [5]. While this leaves open the question of a role for these SLRPs in later tumour progression, the implication of altered expression for the earlier stages of tumourigenesis remains intriguing. It is possible to speculate that both induction of lumican and decrease in decorin in stromal fibroblasts within the invasive tumour may represent a positive host response, to abrogate the disorganization of collagen within the tumour stroma, encourage macrophage localization [21], and inhibit the growth of epithelial cancer cells, through the increased availability of growth factors inhibitory to breast epithelial cell growth [22]. Alternatively, these alterations may represent a negative host response contributing to early tumour development. Increased lumican mRNA expression may reflect a response to locally increased proteolysis or altered deposition of the lumican protein that is the cause of the disorganization of the collagenous stroma, which in turn facilitates tumour cell invasion. Similarly, a decrease in decorin may remove an inhibitory effect on epithelial tumour cell growth through repression of p21 [7]. A role for and

the distinction between these opposing potential effects will clearly require further study.

The differences in lumican levels between normal and tumour tissues observed by both immunohistochemistry and western blot are not as marked as those seen at the level of mRNA expression. While differences in the assays may account for some of this discrepancy, it is clear that it may also be attributable to the discordance that can exist between lumican mRNA and protein expression detected by *in situ* and immunohistochemical techniques respectively, within the same regions of breast tumour stroma. A similar discordance between mRNA and protein expression has been previously observed in the course of studies on lumican and other large and small proteoglycans in different tissues. For example, in corneal development in the chicken, the mRNA levels for lumican and decorin do not always reflect the rate of synthesis of the corresponding proteins and the efficiency of translation of lumican varies over time [23]. Similar discordance between aggrecan and versican mRNA and protein has been seen in normal tendon [24], between decorin and biglycan mRNA and protein localization in normal and reactive gastric mucosa [25],
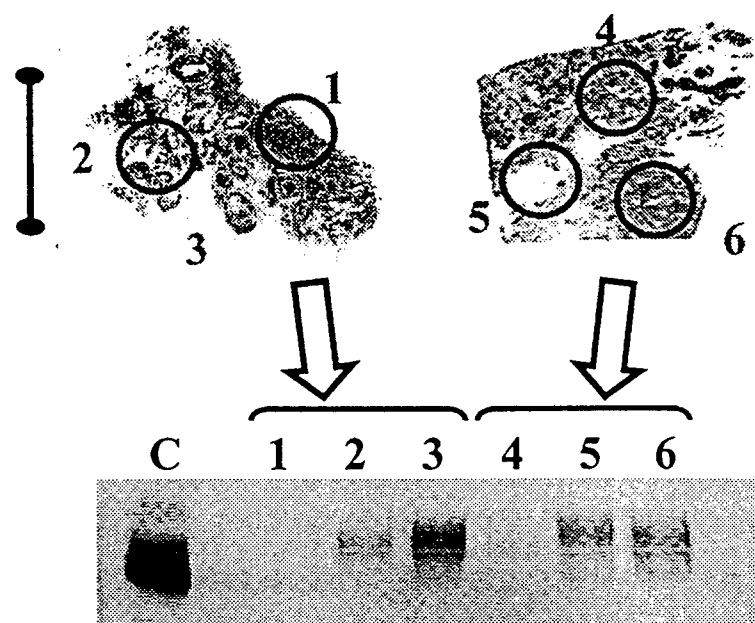
Figure 5. Lumican protein expression detected by immunohistochemistry (upper panel) and western blot (lower panel) demonstrating concordance in the assessment of protein levels in microdissected subregions within two breast tumour sections. The upper panels show IHC sections (tumour A, right; tumour B, left; scale bar =5 mm). The mRNA and protein signals were detected by in situ hybridization (ISH) and immunohistochemistry (IHC) in each region in adjacent sections and ISH/IHC levels were assessed semi-quantitatively (negative, weak +, strong + +) as follows: tumour A: region 1 = + +/−, region 2 = −/+ +, region 3 (remainder of section) = +/+ +; tumour B: region 4 = +/−, region 5 = −/+ +, region 6 = + +/+ +. The lower panel shows the western blot (C= cartilage control; lanes 1–6 correspond to regions assessed and microdissected above)

and in regions of cartilage matrix around vascular channels and the growth plates of long bones in normal cartilage [26]. In this latter instance, the discordance was attributed to a high rate of breakdown and removal at these sites. This conclusion is supported by studies on endothelial cells which show that growth factors such as bFGF can increase not only both biglycan transcription and protein synthesis, but also the corresponding rate of proteolysis [27]. The absence of protein could also reflect masking of the epitope by conformational changes in the native protein, by changes in post-translational modification, or by binding to another protein. Alternatively, this could reflect reduced translation, increased breakdown, or failure to bind within the immediate stroma and rap translocation of the protein to adjacent areas of the tissue. Our microdissection experiments, applied to small regions where lumican mRNA is highly expressed, suggest that the corresponding protein is truly absent in these areas and that epitope masking due to conformation or binding proteins is an unlikely explanation for the observation. However, it could also be the case that the necessary binding sites are not available in the immature stroma associated with rapid growth of tumours and that this allows translocation of newly synthesized lumican to binding sites in adjacent tissue.

The reciprocal nature of the changes in the expression of lumican and decorin is intriguing. Although definitive characterization of the stromal cell types awaits primary culture studies, direct comparison of in situ hybridization performed on serial sections suggests

that expression of both genes apparently occurs in the same fibroblast-like cells in breast tissue stroma. While lumican has not previously been studied in human tumours, the expression of decorin mRNA and proteoglycans incorporating chondroitin sulphate epitopes has been shown to be increased in colon, prostate, and basal cell carcinomas [28–30], but a more recent study of multiple stromal genes in breast tumours found no difference in the levels of decorin mRNA between tumour and normal tissue, although noting increased expression in the stroma immediately adjacent to in situ components [31]. However, the normal tissue examined was selected to be well away from the primary tumour and this, together with differences in the method of quantitation, the definition of tumour regions, and the focus on matched samples, limits a full comparison with our observations. For example, morphologically normal tissue immediately adjacent to carcinomas may be influenced by paracrine growth factors derived from the tumour and may also harbour molecular alterations [32] that might influence local gene expression. However, similar immunohistochemical studies of breast tumours using monoclonal antibodies raised against chondroitin sulphate and dermatan sulphate small proteoglycan have shown reduced decorin expression within invasive as compared with surrounding normal stroma, consistent with our findings [33]. Decorin and other SLRPs are known to be independently regulated and mutually exclusive [26] and compensatory changes in the expression between different SLRPs have been observed [34]. However, this appears to be usually

manifested by genes within subgroups of the SLRP family. At the same time, reciprocal changes in the expression between lumican and decorin have not been described in lumican or decorin 'knockout' mice [8,17]. The factors that influence altered expression of these genes in breast tumour stroma remain to be elucidated.

In summary, we have shown that lumican is highly abundant relative to decorin, biglycan, and fibromodulin in normal and neoplastic breast tissues. We have also shown that increased lumican protein expression and altered regional localization occur in breast tumours and that different and reciprocal alterations in expression occur between lumican and decorin. The functional significance and the role of alterations in these stromal proteoglycans in breast tumourigenesis and progression remain to be determined.

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Interaction of the Differentially Expressed S100A7 Gene With Centrosomal Proteins. Ethan D. Emberley*, A. Kate Hole, R. Daniel Gietz, Leigh C. Murphy and Peter H. Watson. Pathology, University of Manitoba, Winnipeg, Manitoba, Canada; and Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba, Canada.

We previously utilized a subtraction hybridization technique to identify the S100A7 (psoriasin) gene as being differentially expressed between different stages of human breast cancer. Originally discovered as being highly expressed in abnormally differentiated squamous keratinocytes in patients with psoriatic lesions, S100A7 was observed to be a secreted protein with chemotactic ability for $T4^+$ lymphocytes. We recently used In-situ hybridization, RT-PCR, IHC and Western blot to confirm high S100A7 expression in DCIS compared to normal and invasive breast tissue. Protein expression was also found to be present in both the nucleus and the cytoplasm. However, maintance of protein expression in breast tumors was found to correlate with ER(-) status and poor prognosis.

S100A7 is a member of the S100 family of proteins. Members of this family share numerous similarities such as containing Ca^{2+} binding EF-hand domains, location in a gene cluster at position 1q21 and a molecular mass of about 10 KDa. Currently, there are no data on the biological role of S100A7, and how its altered expression contributes to the biological process of invasion. To address this question and identify potential pathways or cellular processes in which S100A7 participates, we utilized the yeast two-hybrid assay to identify interacting proteins. Full length S100A7 was used to screen 17.3×10^6 clones from a normal human mammary cDNA library. Two centrosomal proteins, RanBPM and hGCP3, were determined to be true positives in the yeast assay and were selected for further study. As well, confocal microscopy analyses show that S100A7 does indeed localize to the centrosome. The biological importance of these interactions is under investigarion.

Disclosure: No disclosure information to disclose

Signature: _E.Emberley_
(Ethan D. Emberley B.Sc. Honors)

Date: June 1 /00

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